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# **Association between BCG-induced immunity and risk of TB disease**

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**Declaration**

I, Benjamin Mugo Njeru Kagina, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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**Summary: Association between BCG-induced immunity and risk of TB disease. Benjamin M. N. Kagina, 2<sup>nd</sup> June, 2010**

Tuberculosis is a pandemic disease. The incidence of the disease is particularly high in infants. The current vaccine against tuberculosis is therefore administered soon after birth. BCG protects against severe infant tuberculosis, but has variable and mostly poor protection against pulmonary disease. Consequently, development of more effective vaccines that protect against pulmonary disease is a priority.

We tested the hypothesis that a lower frequency and profile of specific T cells induced by BCG vaccination at birth is associated with subsequent risk of developing tuberculosis. Whole blood from 10-weeks old infants, routinely vaccinated with BCG at birth, was incubated with BCG for 12 hours, followed by cryopreservation of white cells. Infants were followed for 2 years to identify those who developed culture-positive tuberculosis (cases), and those who did not, despite exposure to tuberculosis in the household (controls). Another group of randomly selected controls, never evaluated for TB, were also identified. Cells from cases and controls were thawed, and using flow cytometry, T cell-specific expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-17 was measured. The BCG-specific T cell cytokine frequencies and profiles did not associate with risk of developing tuberculosis. We concluded that critical components of immunity against tuberculosis, such as IFN- $\gamma$  production by CD4 T cells, may not translate to correlates of risk of tuberculosis.

We also tested the hypothesis that delaying BCG vaccination from birth to 10-weeks of age results in induction of more optimal specific T cell immunity. The rationale is that we may be able to use BCG more optimally by later administration, given the relative “immaturity” of the newborn’s immune system. BCG was either administered at birth or at 10 weeks of age. Ten weeks after vaccination, and at 1 year of age, vaccine-specific T cell expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-2, were measured with the same whole blood assay. The responding specific T cells were phenotyped for memory markers. Infants who received delayed BCG vaccination demonstrated



higher frequencies of BCG-specific CD4 T cells, particularly polyfunctional T cells co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, and most strikingly at 1 year of age. We concluded that BCG might be used more optimally, if given later than immediately after birth.

The findings in this thesis are contributing significantly to the field of tuberculosis vaccinology. For example, new approaches to delineating host correlates of risk of tuberculosis are needed, and the effect of age of giving BCG vaccination on efficacy should be studied further.

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## List of Abbreviations

A	Adenine
Ab	Antibody
AIDS	Acquired Immuno Deficiency Syndrome
APC	Allophycocyanin
APCs	Antigen presenting cells
ATP	Adenosine Triphosphate
BCG	Bacillus Calmette-Guérin
BIS	Brightness index score
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
CCR	Chemokine receptor
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CFUs	Colony forming units
CMV	Cytomegalovirus
CO <sub>2</sub>	Carbon dioxide
CoR	Correlates of risk
CPT	Cell preparation tube
CTLA	Cytotoxic T-lymphocyte antigen
CV	Coefficient of variation
Cy	Cyanine
dH <sub>2</sub> O	Distilled water
DC	Dendritic cell
DC SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- integrin
DMSO	Dimethyl sulfphoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment strategy
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot

EPI	Expanded program on immunization
ESAT-6	6 kDa early secretory antigenic target
FITC	Fluorescein
FCS	Fetal calf serum
FMO	Fluorescent minus one
FSC	Forward scatter
G	Guanine
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
IFN- $\gamma$	Interferon gamma
IL	Interleukin
IGRAs	Interferon gamma release assays
IPP	Isoprenyl pyrophosphate
iTreg	Induced regulatory T cells
LAG	Lymphocyte-activation gene
LTBI	Latent tuberculosis infection
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
MDR	Multidrug resistant
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
Min	Minute
mL	Mililitre
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
<i>M.vaccae</i>	<i>Mycobacterium vaccae</i>
NADH	Nicotinamide adenine dinucleotide
NK	Natural killer
NO	Nitric oxide
NRAMP1	Natural resistance associated macrophage protein 1
NTM	Non-tuberculous <i>mycobacteria</i>
Pac	Pacific
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	R-Phycoerythrin
PFC	Polychromatic flow cytometry
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophils
PMT	Photomultiplier tube
PPD	Purified protein derivative of <i>M.tb</i>
PWM	Pokeweed mitogen
QDot	Quantum dot
RAG	Recombinase-activating genes
rBCG	Recombinant Bacillus Calmette-Guérin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SATVI	South African Tuberculosis Vaccine Initiative
SEB	<i>Staphylococcal enterotoxin B</i>
SIV	Simian immunodeficiency virus
SLC11A1	Solute carrier family 11 member a1 gene
S/N	Signal to noise ratio
SNP	Single nucleotide polymorphism
SSC	Side scatter
TB	Tuberculosis
T <sub>C</sub>	Cytotoxic T cells
T <sub>CM</sub>	Central memory T cells
TCR	T cell receptor
T <sub>EM</sub>	Effector memory T cells
T <sub>EMRA</sub>	Terminally differentiated memory T cells
TGF-β	Transforming growth factor β
T <sub>h</sub>	T helper
TIM	T cell immunoglobulin mucin
T <sub>NAIVE</sub>	Naive T cells

TNF- $\alpha$	Tumor Necrosis Factor-alpha
Treg	Regulatory T cells
TST	Tuberculin skin test
Uns	Unstimulated
WHO	World health organization
XDR	Extremely Drug Resistance
$\gamma\delta$ T	Gamma delta T cells
$\mu$ L	Microlitre

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I thank you Lord for protecting and empowering me this far. Help me to use the knowledge acquired to lessen the global TB morbidity and mortality.

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## **Aims**

- (i) To assess whether long-term cryopreservation would allow reliable measurement of mycobacteria-specific T cell response (Chapter 2)
- (ii) To optimize a polychromatic flow cytometry panel for the measurement of BCG-specific T cells in infants (Chapter 3)
- (iii) To evaluate the association between the frequency and cytokine expression profiles of antigen-specific T cells with the risk of childhood TB disease, following BCG vaccination at birth (Chapter 4)
- (iv) To assess the effect of delaying BCG from birth to 10 weeks of age on the BCG-specific T cell response (Chapter 5).

## **Chapter 1: General introduction**

### **1.1 What is tuberculosis?**

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (*M.tb*). TB disease mainly occurs in the lungs although it may disseminate to other organs (1). TB diseased persons cough, allowing airborne transmission to others, who inhale the pathogen. Infants and children younger than four years are at greater risk of developing TB disease after primary *M.tb* infection, compared with older children, adolescents and young adults (2, 3). Therefore, this thesis has focused on TB immunology studies in infants.

### **1.2 Epidemiology of tuberculosis**

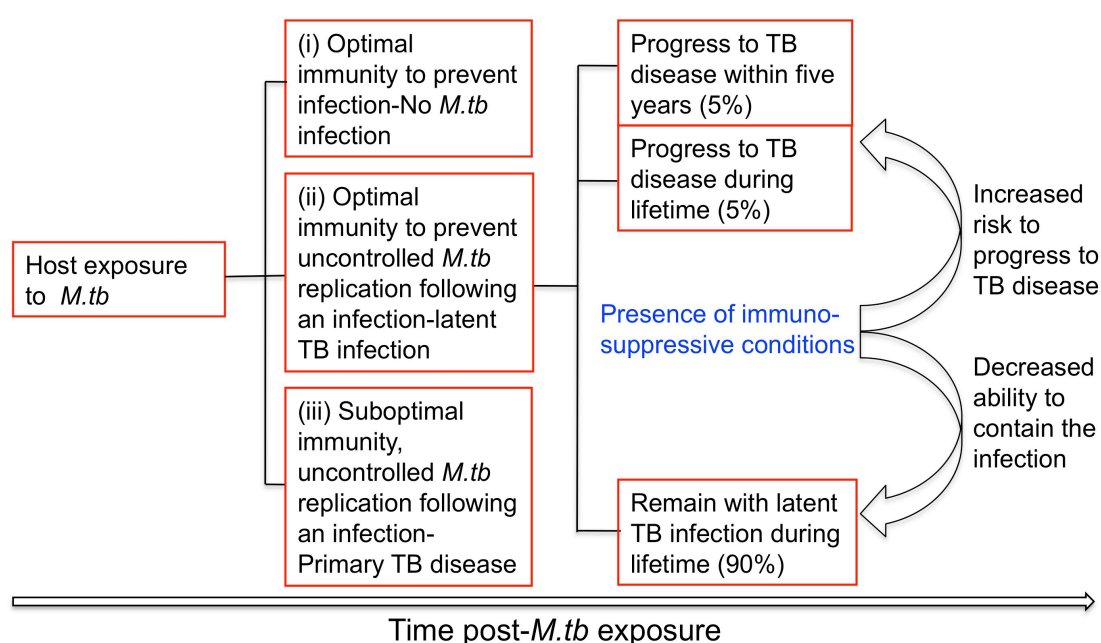
In 2008, the World Health Organization (WHO) estimated a TB incidence of 139 cases per 100,000 populations, globally (4). Africa and Asia had the most number of TB cases globally (4). Based on TB incident rates, 22 high burden countries have been identified by WHO, and South Africa is ranked fifth on the list of these countries (4). In South Africa, the Worcester region of the Western Cape has a TB incidence in excess of 1,000/100,000/year among children <2 years of age (5).

The South African Tuberculosis Vaccine Initiative (SATVI) established a vaccine evaluation site in the Worcester region in 2000. Our aim was to study new TB vaccines, and address clinical, epidemiological and immunological questions that are critical for TB vaccine development. Some of the studies reported in this thesis were performed on infant participants recruited from the site.

### **1.3 Natural history of tuberculosis**

Tuberculosis diseased persons expel *M.tb* into the air when they cough or sneeze. The bacterium is contained in the aerosol droplets. Healthy individuals who inhale *M.tb* contained in the aerosol microdroplets may: (i) resist infection, (ii) become infected and develop TB disease (primary TB), (iii) become infected and contain the infection (latent TB), and (iv) become

latently infected, and later progress to TB disease (secondary or reactivation TB) (6). In absence of human immunodeficiency virus (HIV) infection or any other immunosuppressive condition, it is estimated that 5% of latently *M.tb* infected people progress to develop active TB within five years and another 5% reactivate to active TB during their lifetime (7). If no preventative measures are implemented, infants below one year of age have the highest risk of progressing to TB disease following primary *M.tb* exposure; up to 30% develop pulmonary or disseminated TB within one year (2). **Figure 1** illustrates the possible outcomes of a host following *M.tb* exposure.



**Figure 1:** The possible outcomes of host exposure to *M.tb*: (i) Some individuals successfully clear the infection, (ii) others contain the infection, (iii) while the remaining individuals develop primary TB disease. Differences in innate and adaptive immunity may account for these potential outcomes. Five-percent of the latently infected individuals progress to secondary TB disease within five years while another five-percentage progress to TB disease during their lifetime. In the presence of immunosuppressive conditions, the risk of progressing to TB disease is increased. The figure is adapted from Bhatt *et al.*(8).

### 1.3.1 Risk modifiers to tuberculosis natural history

Several risk modifiers may play a role in the natural history of TB within a host. These risk modifiers include:

- (a) Host genetic factors. Some genetic determinants have been linked with susceptibility to *M.tb* infection and subsequent progression to TB disease, e.g., in a South African population of mixed race, Barreiro *et*

*al.* found that a single nucleotide polymorphism (SNP) of CD209 at two alleles; -871G and -336A was associated with decreased TB susceptibility (9). Also, among Chinese patients with coal worker's pneumoconiosis, presence of a SNP in the tumor necrosis factor (TNF- $\alpha$ )-308A allele was reported to be associated with an elevated risk for pulmonary TB (10). Among others, susceptibility to TB may also be influenced by genetic variants of the major histocompatibility complex (MHC), the macrophage protein solute carrier family 11 member a1 gene (SLC11A1; also known as natural resistance associated macrophage protein, NRAMP), receptors to vitamin D, molecules in the interferon gamma (IFN- $\gamma$ )/interleukin twelve (IL-12) pathway, as well as molecules in nitric oxide synthase pathways (11-14).

- (b) Age. The neonate's immune system is less "mature", compared with that of adults (15-18). Consequently, newborns are more susceptible to multiple infections, including *M.tb*. Independent of factors such as HIV/AIDS, environment, genetics or social-economic status, infants and children are at higher risk of developing TB disease after *M.tb* infection, as mentioned above (2). In South Africa, TB disease case notification rates was shown to be 3.5 times higher in 0-5 year old children than in adults (19). Thus, infants should be a target population for vaccination with an effective TB vaccine. Assessment of correlates of risk of TB disease in infants may hold clues to critical aspects of immunity that predispose infants to greater risk of development of TB.
- (c) Immune suppressive conditions. Persons with immune suppressive conditions such as diabetes mellitus and HIV/AIDS are more vulnerable to established *M.tb* infection and subsequent TB disease development (20, 21). One possible explanation to vulnerability in *M.tb*/HIV co-infected persons is the HIV-associated apoptosis of CD4 T cells (22), a subset of immune cells that has been shown to be necessary for protective immunity against TB (23). A study by Hesselning *et al.* in Western Cape, South Africa, reported that HIV-

infected infants were 24 times more likely to develop TB than HIV-uninfected infants (20).

- (d) Socio-economic factors. A strong association was reported between homelessness and the incidence of TB in a community living in the northern region of Poland (24). A positive relationship between crowded environments (25), poverty (26) with TB incidence has been reported.

#### **1.4 Interventions against tuberculosis**

In response to the morbidity and mortality associated with TB, the WHO together with various stakeholders has suggested multi-pronged interventions:

- (a) TB diagnosis. Sensitive and rapid diagnosis of TB is critical for control of TB pandemic. Clinical, radiological as well as epidemiological scoring systems, sputum microscopy, detection of *M.tb* cellular components by molecular methods, and detection of host-specific immune response to *M.tb* are some of the main laboratory methods of diagnosing TB (27-30). Tuberculin skin test (TST) and IFN- $\gamma$  release assays (IGRAs) detect the host immune response to *M.tb* antigens, and are widely used in the diagnosis of latent TB. The TST assesses the host delayed hypersensitivity reaction, characterized by inflammation of the dermis at the site of purified protein derivative (PPD) injection. *M.tb* PPD consists primarily of membrane proteins derived from this pathogen, but these proteins are very similar across mycobacterial species (31). Consequently, the TST response may also be positive in individuals previously vaccinated with BCG as well as individuals exposed to environmental mycobacteria (32). On the other hand, IGRAs measure the host IFN- $\gamma$  responses following incubation of whole blood with *M.tb*-specific antigens of the region of difference (RD-1) that includes ESAT6, CFP10 and TB7.7, by enzyme-linked immunosorbent assay (Elisa) or enzyme-linked immunosorbent spot (Elispot) (33). Therefore, IGRAs are more specific and have a better correlation with epidemiological risk factors



for TB infection than TST, at least in low-prevalence countries (34, 35). In children less than five years old, the reliability of IGRAS has not yet been determined (36), especially not in the presence of malnutrition and immune deficiency. Malnutrition and HIV associated immune deficiency are most prevalent in TB endemic countries (37) where timely diagnosis is much needed. The gold standard for diagnosing TB disease is bacteriological confirmation in sputum samples (29). The sensitivity of sputum microscopy and culture to diagnose TB disease in children is compounded by the pauci-bacillary nature of childhood TB and the difficulty of obtaining these samples for culture (27). Therefore, clinical and radiological features are often used to make the diagnosis in this population; many of these markers are non-specific.

- (b) TB treatment. The WHO target for successful treatment is a cure rate of 85% of the 70% TB cases detected (38). Optimal implementation of the directly observed treatment strategy (DOTS), introduced by WHO in the mid 1990s (39), is key to achieving treatment targets. The principle of treating children and adults for TB is similar (40). Drugs used in the treatment of TB are classified as first-line, second-line or third-line mainly, based on efficacy and drug sensitivity (41). Treatment can either be to prevent latent TB from progressing to disease, or to treat TB disease itself (42). Treatment of latent TB in children involves first line drugs like isoniazid for 6 months or rifampicin and isoniazid for 3 months (43). This treatment is well tolerated (44) and can achieve 60-80% efficacy in prevention of TB disease (45). To achieve microbiological cure of drug susceptible *M.tb* in patients with TB disease, multiple first-line TB drugs must be taken daily for at least 6 months (46). Compounding this protracted TB treatment is the rapid resolution of clinical symptoms prior to the required period of TB chemotherapy (47). Consequently, some patients do not strictly adhere to the anti-TB drug treatment schedule, which may lead to outgrowth of resistant organisms (48). TB patients with multiple drug resistance (MDR) *M.tb* strains are treated with the second-line of drugs such as neomycin, ciprofloxacin and

capreomycin (41). These second-line drugs are more toxic and expensive than first-line drugs (41). Third-line drugs such as rifabutin, clarithromycin and linezolid are used for treating patients with extensively drug resistant (XDR) *M.tb* strains, and are even more toxic and expensive than second-line drugs (41).

- (c) TB vaccines. BCG is the only vaccine currently available for use to prevent TB. The vaccine is effective against severe forms of TB in children (49). However, BCG is not very effective at preventing pulmonary TB, the most prevalent form of the disease (50). There is a general consensus among health experts that an effective vaccine against TB would be the most pragmatic approach in addressing the pandemic. Abu-Raddad *et al.* used a mathematical model to show that if all the novel TB interventions (drugs, vaccines, diagnostics) currently in development were successful, the greatest reduction in TB incidence would be achieved through effective neonatal vaccination (51). Therefore, development of more effective TB vaccines against the pandemic is a priority in TB intervention list. New TB vaccines may either be preventive or therapeutic (52). Among preventive vaccines, prime vaccines are intended for use in newborns and infants prior to *M.tb* or environmental NTM exposure. BCG, modified BCG and attenuated *M.tb* are examples of this type of vaccine (52). Knowledge of variables such as age of vaccination, dose as well as the type of immunity elicited by the prime vaccines is vital for their optimal use. Chapter five studies in this thesis evaluated the effect of delaying BCG to 10 weeks of age on the BCG-induced immunity. In the absence of known immune correlates of protection against TB, the optimal age to give the second type of preventive vaccine, a boost vaccine, is not known. Establishing the optimal age to give the boost vaccine is still an active area of research. Nevertheless, epidemiological data indicate the administration of booster TB vaccines may be ideal during the first two years of life and early in adolescence; TB incidence has been shown to peak in infancy and during adolescence (2, 19). Boost vaccines classically consist of specific mycobacterial antigens delivered in specialised vectors, or with T helper I (Th1)-inducing

adjuvants. Novel therapeutic TB vaccines would be given to individuals with active TB. These vaccines would complement the antibiotic treatment, thereby shortening the duration of anti-TB chemotherapy. Proposed candidate therapeutic TB vaccines include inactivated mycobacteria and DNA subunit vaccine encoding for mycobacterial heat-shock proteins (52).

- (d) Public health interventions. TB diseased persons who are not taking anti-TB drugs spread the disease. Rapid identification and treatment of these infectious persons within a population is therefore central in the control of the disease. Upon initiation of TB chemotherapy to control infectious cases, DOTS is integral in ensuring patients adhere to the treatment schedule. In most countries, national TB programs have been established to coordinate TB control efforts. The success of national TB programs to achieve timely TB case detection, treatment as well as adherence to the treatment is dependent on optimal national health system (53). Bottlenecks cited with suboptimal health systems include lack of adequate health workforce, financing, drug supply, information systems and governance (53). Reforms geared at addressing these bottlenecks in health systems have been shown to significantly improve national TB control programs, and subsequently reduce the disease burden (53). For example, Wang *et al.* reported significant improvements in TB control in China, largely due to reforms of the national health care system (54). In South Africa, health sector reforms that allowed for the creation of district health system were reported to have substantial success in the management of TB (55).

### **1.5 Success of *Mycobacterium tuberculosis* in the face of the current vaccine**

Despite decades of global and national TB control programs as well as BCG still being one of the most widely used vaccine within the expanded program of immunization (EPI) (56), (in 2008, BCG had over 80% global vaccination coverage) (57), global TB burden remains unabated (58). The success of

*M.tb* to cause human morbidity and mortality can partly be attributed to several survival mechanisms the bacilli has adopted to survive in the hostile host environment. Many *M.tb* survival strategies have been described, including the DNA repair mechanism that involves DnaE2 enzyme, restoring damage to the bacterial DNA caused by the host immune defenses (59). Another survival mechanism of *M.tb* is the ability to arrest phagosome maturation through mechanism of pH homeostasis within the macrophages (60). This allows the intracellular *M.tb* to undergo uncontrolled growth. *M.tb* has also been shown to express nicotinamide adenine dinucleotide (NADH)-dependent peroxidase and peroxynitrite reductase that detoxify reactive oxygen species (ROS) and reactive nitrogen species (RNS), produced by the host antigen presenting cells (APCs) (61). This renders *M.tb* resistant to the host antimicrobial components. Development of new vaccines would therefore not only require identification of correlates of protection, for example, but also intensive basic science research into mechanisms to circumvent bacterial evasion of the host response. Multiple drugs and vaccine candidates that specifically address this issue are under development (62-64).

## **1.6 Host immunity to *Mycobacterium tuberculosis***

### **1.6.1 Innate immune response to *Mycobacterium tuberculosis***

Interaction of the inhaled *M.tb* and the host immune cells begins in the alveoli, tiny sacs in the lungs specialized in gaseous exchange. In the alveoli, APCs which include alveolar macrophages, dendritic cells (DCs) and polymorphonuclear neutrophils (PMNs) patrolling the distal airways may directly phagocytose the bacilli (65). The uptake of the bacilli by a host APCs is via phagocytic and recognition receptors (66-68). These receptors among many others include mannose-containing glycoconjugates, Fc gamma receptor, complement receptor, C-type lectins such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC SIGN) and dectin-1, toll like receptors (TLR), CD43 and CD14 (69-72). Differential receptor interaction will result in distinct functional consequences, such as phagocytosis or biological processes in the cell, such as DC maturation,

cytokine or chemokine production or MHC expression (73, 74). It is thought that the initial interaction between *M.tb* and the host APCs determines the subsequent immune response. This is partly because of differential induction and constitutive expression of recognition and phagocytic receptors on various APCs (75).

The PMNs are short-lived and undergo apoptosis soon after phagocytosis of the *M.tb* (76). In a mouse model of virulent *M.tb* challenge, depletion of PMNs resulted in a decreased recruitment of specific Th1 cells (77). This suggests that these cells (PMNs) may also play a critical role by producing chemokines that attract other mycobacteria-specific cells to the site of infection. *In vitro* experiments have shown that human PMNs can inhibit growth of both BCG and *M.tb*, via production of antimicrobial peptides such as cathelicidin LL-37 and lipocalin 2 (78). Some alveolar macrophages may also undergo apoptosis following increased burden of the *M.tb* in the phagosome (79, 80). The apoptotic APCs are taken up by secondary alveolar macrophages and DCs. Once phagocytosed by the DCs or secondary alveolar macrophage, the bacilli are again contained within phagosomes (81, 82). Knowledge on the killing mechanisms of the intracellular *M.tb* by the host APCs in humans remains incomplete. However, in animal models, it has been shown that, the bacilli in the phagosome may be killed by antimicrobial components that include lysosomal enzymes, ROS, siderocalin and RNS (83-85). Autophagy provides an additional route for the elimination of the intracellular *M.tb* by the host APCs (86, 87). In this process (autophagy), intracellular *M.tb* escaping into the cell cytoplasm may be sequestered together with other cellular cytoplasmic entities, and then targeted for degradation by fusion with lysosomes (88).

Natural killer (NK) cells are also thought to be important in mediating immunity against *M.tb* infection. Experimental data show that NK cells can recognize and lyse *M.tb* infected monocytes and macrophages through production of cytotoxic molecules (89). Furthermore, NK cells produce cytokines, mainly IFN- $\gamma$ , that can shape the adaptive response to *M.tb*. (90). If not killed, the intracellular *M.tb* may then undergo a growth phase within

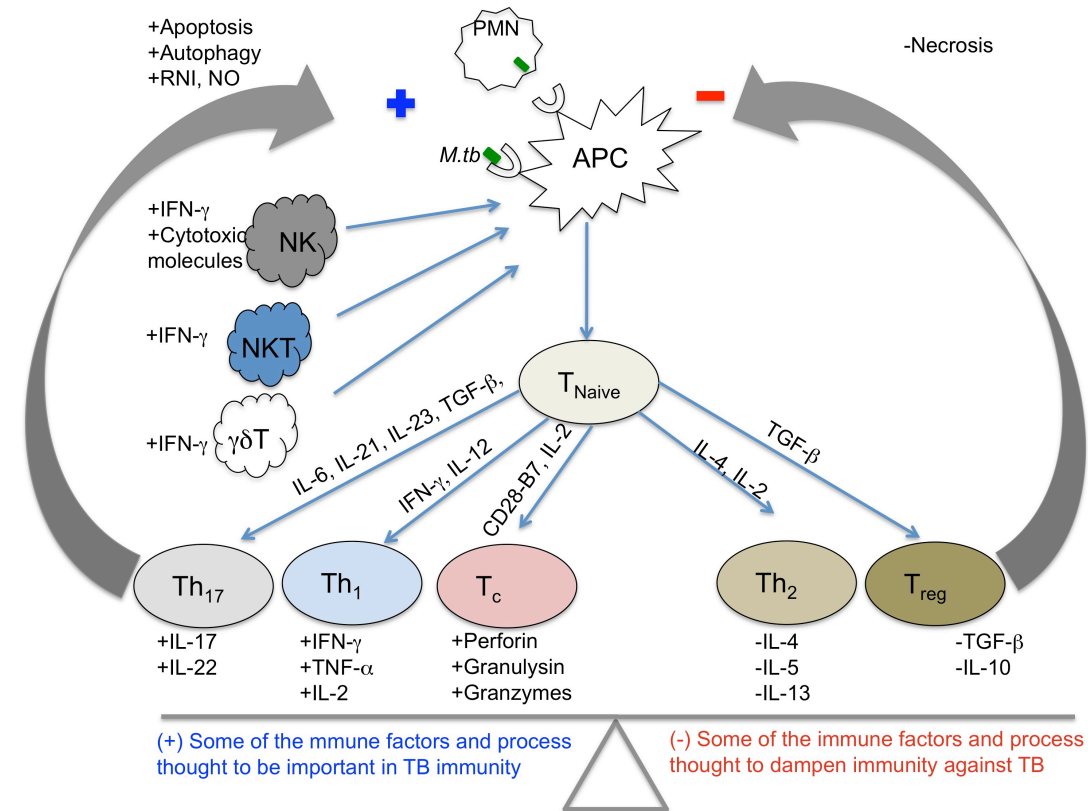
the APCs to establish an infection. If not killed, the intracellular *M.tb* may then undergo a growth phase within the APCs to establish an infection. Inability by the host immune response to control the *M.tb* replication results to development of clinical TB disease whereas the ability by the host to control the *M.tb* replication results to sub clinical TB. The innate system alone may not be adequate to control the infection – help from T cells, which activate the phagocytes, may be necessary (see below). Essentially, innate immune cells constitute the first line of defense by the host immune system against *M.tb*.

### **1.6.2 T cell immune response to *Mycobacterium tuberculosis***

Dendritic cells infected with *M.tb* migrate from the site of infection to the draining lymph nodes and present the *M.tb* peptides to cognate T cells to initiate a second line of host defense, which is cell mediated immunity (91, 92). These T cells respond by producing cytokines such as IFN- $\gamma$  that activates the APCs to enhance control of the intracellular *M.tb* (93). Additionally, the activated T cells produce cytotoxic molecules such as perforin, which mediate lysis of *M.tb* infected cells, or granulysin, which may kill mycobacteria directly (94, 95). Thus, a positive loop exists whereby the activated APCs produce cytokines such as IL-12 that drive differentiation of Th1 cells while T cells produce IFN- $\gamma$  that activate the APCs. It is presumed that optimal innate and optimal adaptive immunity act synergistically to limit the bacilli growth by formation of granulomas in the lungs. Granulomas comprising macrophages, epithelioid cells, multinucleated giant cells, and lymphocytes, are the pathologic hallmark of TB, and are thought to contain *M.tb* infection (96, 97). Conversely, suboptimal innate and adaptive immunity may lead to poor granuloma formation, resulting in spread of *M.tb* infection and subsequent development of TB disease. The exact immune factors and mechanisms that lead to the successful or unsuccessful local immune response to *M.tb* infection are poorly understood. Additionally, immune correlates of protection against TB are not known. The identification of the correlates and biomarkers of protection against TB may accelerate the development of novel effective TB vaccines because scientists would be able

to design a vaccine with properties that can induce the desired immunity. In view of guiding novel TB vaccine development and testing, our chapter four studies investigated the correlates of risk against TB disease in newborns following BCG vaccination at birth.

### 1.6.3 Summary of immunity to *Mycobacterium tuberculosis*



**Figure 2: Immune response and regulation during *M.tb* infection.** Molecules shown on the left side with a “+” sign are thought to mediate immunity during *M.tb* infection while molecules shown on the right side with a “-” sign are thought to regulate the response. Optimal outcome is thought to result from a balance between the two processes.

### 1.7 Immune correlates of protection against TB

Immune correlates of protection are defined as measurable host responses to a vaccine or infectious agent indicating resistance to developing disease (98). Vaccination-induced correlates of protection may be identified in a randomised controlled trial of an effective vaccine; in the interim, we are studying correlates of risk (CoR) of TB disease, with the hope that these will translate into correlates of protection (99).

Investigation of immune CoR of TB disease is only feasible in well-designed cohort studies. Furthermore, it is becoming clear that following *M.tb* infection to a host, a wide spectrum of host responses may underlie the clinical state of “latency” (100). This spectrum needs to be taken into account in studies of CoR. Studies aimed at investigating immune CoR of TB disease begin with optimal selection of study population, definition of TB cases, and delineation of optimal follow up time. Our immune CoR studies involved large number of infants (over 5,000), which is essential for these studies, and utilized qualified diagnostic algorithms to define TB cases, which is also critical (101). Only then can investigators decide which host parameters to assess, and second, how to measure these potential markers. From our current understanding of *M.tb* immunobiology, potential CoR of TB disease may be classified into 2 groups: factors associated with too much inflammation, and factors associated with excessive anti-inflammatory responses and excessive regulation of potentially protective effector responses. Pro-inflammatory factors may be associated with innate cells, including production of IL-1, IL-6, IL-12, or associated with T cells, such as effector Th1 or Th17 responses, or cytotoxic activity (102); excessive activity of these responses, which may all be important for protection, could be deleterious. Therefore, anti-inflammatory factors produced by innate cells, such as IL-10, and even Th2 responses or Treg activity may be important. Our focus in this thesis was on Th1 and Th17 responses, hypothesizing that optimal responses by these cells, and not excessive or too little, may be associated with protection against TB disease. It should be pointed out that we recognise the potential limitations of this reductionist approach. Therefore, many modern CoR studies have employed unbiased approaches, such as metabolomics, transcriptomics and proteomics, in an attempt to define these host markers that associated with outcome (103).

The importance of identifying immune correlates of protection against TB cannot be overemphasized. First, reliable correlates of protection would guide rational design of novel TB vaccines. Second, the correlates of protection would allow a more focused and standardized immunogenicity evaluation of novel TB vaccines undergoing clinical trials. Finally, when



effective TB vaccines are available and licensed for human use, identification of populations at the highest risk of developing TB disease is feasible, allowing for timely, targeted intervention and subsequent reduction of TB incidence rates.

## **1.8 Vaccine induced immunity against *Mycobacterium tuberculosis***

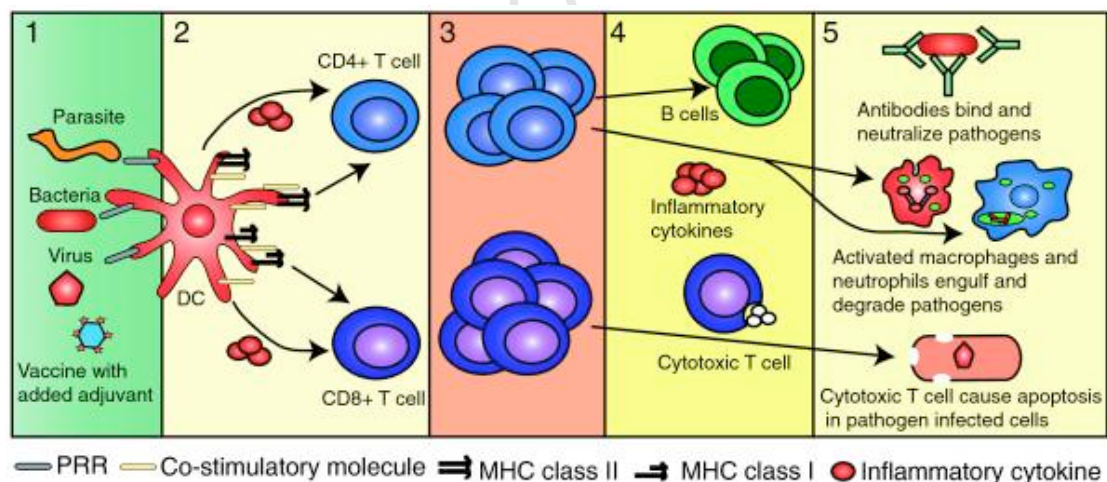
### **1.8.1 Principles of vaccine induced immune protection**

Broadly, immunity can be defined as the ability of the body to protect itself against non-self agents such as pathogens. Vaccination aims to invoke immunity that confers long-term protection against a pathogen. To achieve this, vaccines must contain some or all antigens of the target infectious agent that are recognized by the immune system, for an effective host response. More importantly, the vaccine must be safe. There are three main categories of vaccines used in humans: inactivated pathogens, live attenuated pathogens and subunit vaccines. Vaccine-induced immunity may be B or T cell-mediated, although a combination of the two arms is often required after vaccination (104). T cell immunity is thought to be necessary in conferring protection against *M.tb*. Therefore, majority of new TB vaccines are designed to induce this type of immunity and is the most commonly assessed immune response in clinical trials testing for novel TB vaccines (105, 106).

The vaccine-induced T cell immune response is initiated when antigens contained in the vaccine are taken up by APCs (107). In the peripheral tissues, uptake of the vaccine antigens by immature DCs initiates activation and maturational changes, which are aided by local inflammatory mediators such as cytokines, chemokines and prostaglandins (108). DC maturation involves many morphological and cytoskeletal changes such as upregulation of surface receptors, e.g. chemokine receptors (CCR7 and CXCR4) and costimulatory molecules (CD80 and CD86) (108). The local microenvironment also provides signals required for the DC migration to the secondary lymphoid organs (108, 109). Activated, antigen loaded and matured DCs process the antigens and migrate to the draining lymph nodes to present the antigenic peptides in the grooves of MHC molecules to

antigen-specific naive T cells (107). In the lymph node, antigen-specific naive T cells become activated and differentiate into effector T cells (107). The effector CD4 T cells, and other cells from the microenvironment, provide additional stimuli allowing DCs to polarise into APC providing naive CD4 T cells with signals to differentiate into distinct types of effector T-helper cells, e.g., Th1, Th2, Th9, Th17, and inducible regulatory T cells (iTreg) (109). Therefore, DCs play a critical role in orchestrating vaccine-induced immunity to the targeted organisms.

Antigen-specific effector T cells are short lived (107). When the vaccine is cleared, a small population of clonally expanded antigen-specific lymphocytes is maintained as a memory pool. The memory pool rapidly mounts a protective response upon later, secondary encounter with vaccine directed pathogen (110, 111). **Figure 3** illustrates the possible mechanisms of pathogen/ vaccine-induced immunity.



**Figure 3:** Antigen Presenting Cells (APCs), represented by dendritic cells (DCs) take up the vaccine or pathogen. The DCs become activated and process the complex antigens into peptides. The B cells may also take up antigen and present to T cells (stage 1). Activated DCs present the peptides to cognate CD4 and CD8 T cells (stage 2). In the presence of co-stimulation and cytokines, T cells become activated and proliferate (stage 3). Proliferating T and B cells differentiate into effector cells (stage 4) that can carry out effector functions. Activated CD4 T cells can also provide help to B cells, inducing the production of high-affinity class switched antibody (stage 5). After a successful immune response, effector cells apoptose, leaving a small pool of long-lived memory B and T cells, which is the ultimate goal of vaccination (107).

The necessary vaccine-induced effector/memory immunity against *M.tb* is thought to involve CD4 and CD8 T cells that make type 1 cytokines and have cytotoxic activity (Tc). Pro-inflammatory IL-17-producing T cells (Th17) may also play a role (112-114). Although the role of IL-17-producing T cells in mediating immunity against *M.tb* infection is unclear in humans, experimental data support the importance of these cells in TB immunity. For example, Wozniak *et al.* reported that mice deficient of the IL-12/IFN- $\gamma$  axis developed a robust Th17 T-cell response after BCG immunization, and this response was associated with a significant reduction in the bacterial load following infection with *M.tb* (115). Other investigators have reported that *in vivo* transfer of mycobacteria-specific IL-17-secreting CD4 T cells to susceptible mice [IL-12p40(-/-) or RAG(-/-)] resulted in a significant reduction in the bacterial load following *M.tb* challenge (116). It is likely that optimal vaccine-induced immune responses need to be regulated to avoid immune pathology. Mechanisms that regulate Th1 immunity may include type 2 cytokines (Th2) and regulatory CD4 T cell (Treg) activity (117-120). Among effector T cells, multiple negative regulatory pathways control activation. These involve molecules such as programmed death-1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin mucin-3 (TIM-3), B and T lymphocyte attenuator (BTLA), CD244 and lymphocyte-activation gene 3 (LAG-3), among many others (121, 122). It is likely that a balance between the vaccine-induced immune response and the immune regulatory factors is critical. For example, excessive anti-inflammatory and regulatory factors involving Th2 and Tregs, respectively, may attenuate the protective response (123). This may result in host suboptimal response against *M.tb* infection. It is thought that an interplay and counter regulation of the pro-inflammatory, anti-inflammatory, and regulatory mechanisms influence the outcome of *M.tb* infection within the host. Consequently, evaluation of antigen-specific pro-inflammatory, anti-inflammatory, and regulatory factors could provide key insights to candidate immune correlates of protection against TB. Although it may be therefore important to measure all these aspects of immunity, work in this thesis represents a critical first step, by defining effector/memory T cell immunity.

### 1.8.2 Bacille Calmette-Guérin vaccine in tuberculosis control

Bacille Calmette-Guérin (BCG) is a live attenuated strain of *M.bovis*. The vaccine was first introduced in humans in 1921 and adopted by the WHO in 1948 (124). When administered at birth, BCG affords about 80% protection against miliary and meningeal TB in children (125). In addition, non-specific benefits of neonatal BCG vaccination have been reported (126). These include reduced childhood mortality in infants from low socio-economic environment, not related to protection against TB, protection against other environmental mycobacterial diseases and enhanced immunogenicity and possibly protection afforded by other vaccines given within the EPI (127). The vaccine has good safety record in healthy newborns. BCG is widely used: an estimated 100 million children receive BCG every year (124). However, despite the widespread use of BCG, the global TB burden remains very high: about 1.7 million TB associated deaths occurs annually and one-third of the world's population is asymptotically infected with *M.tb* (128).

Epidemiological data indicates that BCG has variable efficacy against pulmonary TB (50). This efficacy appears to vary in different geographical regions (50), suggesting influence of the environment; genetic factors may also be important. Several hypotheses on immunological explanation to the variability in BCG efficacy have been tested. For example, Peiying *et al.* reported that splenocytes from mice that are presensitised with NTM followed by intranasal BCG challenge exerted a stronger cytotoxic activity against autologous BCG-infected macrophages than mice without NTM presensitisation (129). This suggests that pre-existing host NTM induced immunity may mount an immune response against BCG, resulting to reduced vaccine-induced immunity.

BCG vaccination induces a predominant Th1 response (130, 131). This type of immune response is thought to be important for protection against TB. The administration of BCG vaccine to the host is aimed at mimicking the response to natural and potentially harmful *M.tb* infection. Therefore, the mechanism by which BCG-induced immunity is elicited is thought to be

similar to that following *M.tb* infection. However, BCG is routinely given intradermally, implying the uptake of the vaccine antigens by the host APCs in the intradermal tissue and not by alveolar macrophage, as is the case of *M.tb*. In clinical settings, it is not known whether intranasal or even pulmonary delivery of BCG would elicit a more effective immune response and better protection against *M.tb*. The immunogenicity assays performed during novel TB vaccine clinical trials commonly assess the Th1 response, with presumption that this response may associate with the vaccine efficacy. However, it is not known whether the mycobacteria-induced Th1 immune response translates to protection against development of TB. In this thesis, we assessed whether the age at which BCG is administered may influence BCG-induced immunity. We also assessed if the BCG-induced Th1 immunity would correlate with the risk of developing TB.

#### **1.8.2.1 Factors that may influence BCG-induced immunity**

Factors thought to influence BCG immunogenicity include (i) age at which BCG vaccination is administered, (ii) vaccination route, (iii) pre-exposure to NTM, (iv) BCG strain and (v) host genetic factors.

- a) Age at which BCG vaccination administered: Most TB endemic countries, including South Africa have adopted at birth or soon after birth BCG vaccination schedule. This is in line with the WHO TB control vaccination policy (132). In high TB prevalent areas, the risk of early *M.tb* exposure to infants is high. Also, majority of births occur in health facilities, ideal settings to give BCG and other EPI vaccines. This vaccination schedule results to higher BCG vaccination coverage than if delayed to a later stage. However, the neonate's immune system is adapted towards an *in utero* environment, implying a bias towards a Th2 response (133), and an increased frequency of Tregs (134). Furthermore, some studies have reported reduced functional capacity of neonatal APCs relative to those of adults (135). These characteristics may result in a suboptimal immune response to BCG when administered at birth. A study by Hussey *et al.* reported no differences in BCG-induced immunity when BCG was given at birth or

delayed for 10 weeks as assessed by soluble cytokine levels (136). However, BCG-induced T cell immunity in infants is complex (130), and cytokine co-expression profiles were not evaluated in this study. We therefore proposed to comprehensively assess the effect of age at which the BCG vaccine was given on the vaccine-induced T cell immunity in infants.

- b) Vaccination route: In clinical settings, BCG is administered either percutaneously or intradermally. Factors considered in the choice of administration route may include ease of delivery, costs, consistency of dose delivery, adverse events or immunogenicity. The APCs are the first immune cells to take up BCG in the host. Different types of APCs are compartmentalized within different tissue types of the host (137). Compartmentalized APCs have been shown to have distinct functional capacity (138). It is likely that different BCG delivery routes activate distinct types of APCs. This may result in differential induction of BCG-specific immune responses. Furthermore, it has been shown that intradermal or intramuscular vaccine routes may result in varying immunogenicity against Hepatitis B (139), rabies (140), or influenza viruses (141). Additionally, in orthopoxvirus vaccinia virus challenge mouse model, skin scarification generated superior T cell-mediated immune responses and better protection, compared with intradermal, intramuscular or intraperitoneal vaccinia virus vaccination (142). A study in our laboratory showed that infants vaccinated via these two classical BCG vaccination routes were at similar risk of developing TB (101). Therefore we did not focus our studies to assess for the effects of vaccination route on BCG-induced immunity.
- c) Pre-exposure to environmental non-tuberculous *Mycobacteria* (NTM): Several different strains of NTM have been described (143). Most NTM species have >95% genetic similarity with BCG (144), and are mainly found in soil and open waters (145). It is likely that BCG and most NTM possess similar antigens that induce cross-reactive immune responses. Furthermore, in a mouse model, pre-sensitization with NTM prior to BCG vaccination was shown to reduce the vaccine immunogenicity and protective capacity against *M.tb* challenge (146).

Such preclinical findings on the influence of NTM exposure prior to BCG vaccination have raised concerns related to vaccine immunogenicity in clinical settings with high prevalence of environmental NTM. Coincidentally, a much more reduced BCG efficacy level is reported in countries within the tropics (50, 147). It is known that NTM are highly prevalent in these settings. Weir *et al.* reported that NTM exposure prior to BCG vaccination in Malawian school children did not reduce BCG immunogenicity when assessed by an *in vitro* 6-day whole blood IFN- $\gamma$  response to mycobacterial antigens (147). However, although hypotheses exist, there is still no clear clinical evidence in clinical settings that pre-sensitization of the immune system by NTM, prior to BCG, might reduce or enhance the vaccine immunogenicity. In countries like South Africa where BCG is given at birth before potential exposure to NTM, the vaccine-induced immune responses may be independent of NTM pre-sensitization. In one of our clinical studies where BCG was administered at birth or at 10-weeks of age, collaborators from Stellenbosch University are evaluating whether NTM exposure may have any influence to BCG-induced immunity.

- d) BCG strains: Changes in culture media and colony transfer schedules during the BCG manufacturing process are some of the key changes introduced in order to speed up production of the vaccine (148, 149). It is thought that some of these changes in processing of the vaccine may have resulted in different BCG substrains (150). Currently, over 49 BCG vaccine strains are known to exist, of which 4 are most widely administered worldwide, namely Danish, Japan, Glaxo, and Pasteur (151). Genomic analysis has identified a number of polymorphisms in different BCG strains (150). Some of these polymorphisms are located in genes encoding virulence factors and possibly inducing relevant immune responses (150). Knowledge on whether these polymorphisms impact on BCG-induced immunity is incomplete. In a guinea pig model with *M.tb* challenge after BCG vaccination, Horwitz *et al.* reported no difference in the protective efficacy of six different BCG strains (152). There is no existing clinical evidence to indicate

which BCG strain is more efficacious or induces a more optimal immune response. As such, the choice of which BCG strain to use in different countries may largely depend on factors unrelated to immunogenicity. In July 2000, the South African Department of Health recommended the change of BCG vaccine from the routinely used Japan to Danish strain (153). For the immune CoR studies reported in this thesis, BCG strain (Tokyo 172) was administered randomly to the participants either via percutaneous or intradermal routes (101).

- e) Host genetic factors: Several studies have investigated the role of host genetics in the development of TB. For example, Greenwood *et al.* reported an association between intragenic variants of natural resistance-associated macrophage protein 1 (NRAMP1) gene and susceptibility to mycobacterial diseases in a community of Aboriginal Canadians (154). Susceptibility to TB has been linked to genetic polymorphisms on TLR expressed on macrophages and other leukocytes, and involved in *M.tb* and BCG recognition by the host APC (155). Our laboratory is currently conducting studies to investigate the role mediated by mycobacterial recognition genes on BCG immunogenicity and TB disease outcome.
- f) Host immune deficiency: BCG is a live vaccine that replicates in the host and can cause disease (BCGosis) in an immunocompromised host. In South Africa, among HIV-infected infants, an estimated 110-417 cases of BCGosis are reported per 100,000 BCG vaccinations (156). Furthermore, studies in our laboratory have shown that BCG-induced immunity is severely compromised in HIV-infected infants (157, 158). Such findings have necessitated a policy review by the WHO Global Advisory Committee on Vaccine Safety, who has stated that BCG should not be given to infants known to be HIV-infected (159). Since it is not feasible to accurately determine presence of HIV infection in newborns due to the presence of maternally acquired anti-HIV antibodies, a delay of BCG administration in newborns delivered by HIV-infected mothers should be carefully considered. Findings from our delayed BCG vaccination study reported in chapter five suggest



this vaccination schedule (delay to 10 weeks) should be tested in HIV exposed but uninfected infants.

### **1.8.3 Novel TB vaccines on the horizon**

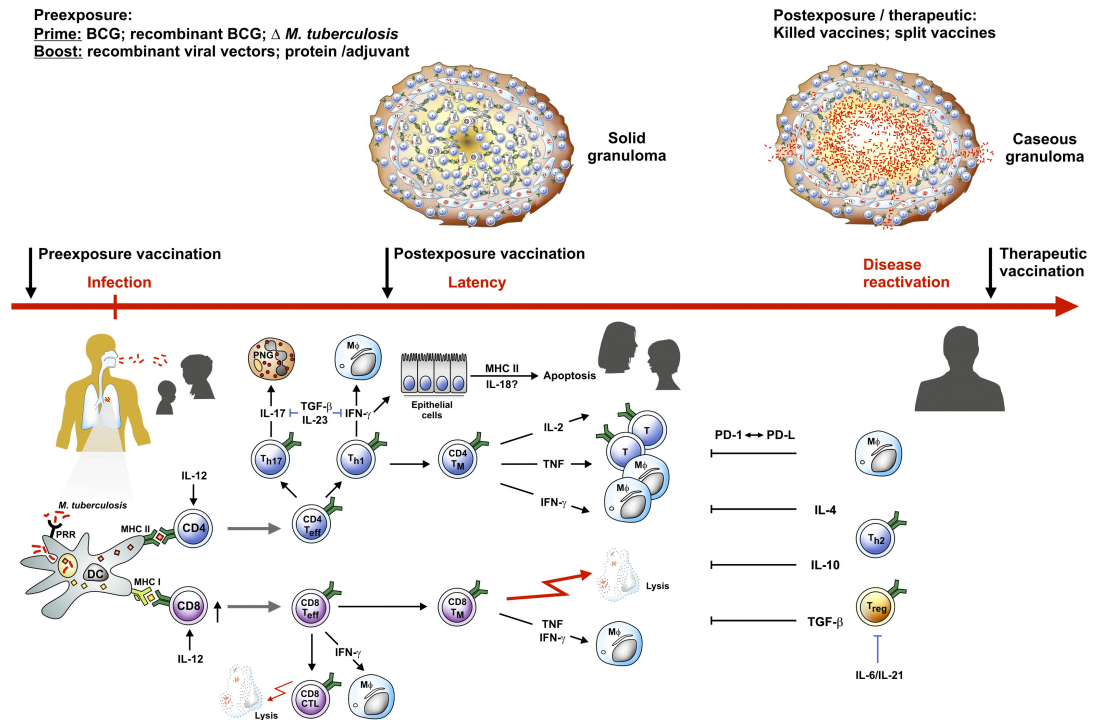
Some vaccines have been shown to be effective through repeated administration with the same vaccine (homologous boost) (160-162). The protective immune response for such vaccines are mainly dependent on humoral immunity. In TB and other mycobacterial diseases, protective immunity is thought to be mainly dependent on cellular immunity and homologous boost has been shown to be ineffective (163, 164). Therefore, prime-boost heterologous vaccination strategy is considered an alternative. The principle of prime-boost heterologous vaccination strategy is to enhance antigen-specific primed immunity. The immunodominant antigens 85A, 85B and TB10.4 have been the primary target for boost following BCG priming. The majority of novel TB vaccines currently in advanced stage of clinical trials are designed to boost BCG-induced immunity (105, 106). The main novel TB boost vaccine candidate are protein-based and viral-based (165).

Modification of the current BCG vaccine to make it more immunogenic is another approach in the development of better TB vaccines. Recombinant BCG (rBCG) vaccines, with increased expression of selected immunodominant antigens have shown promising results at preclinical stage (112, 166). Some rBCG have deletion of a gene encoding for urease in the parent BCG and integration of a gene encoding for listeriolysin (167) or perfringolysin (168). The listeriolysin or perfringolysin gene is associated with lysis of the endosome compartment within the infected cells allowing for enhanced MHC-I antigen presentation (169). Also, endosome escape is associated with induction of macrophage apoptosis; apoptotic cells are cross-presented resulting in enhanced immunity (170). If safety, immunogenicity and effectiveness data compares well against the parent BCG, then, rBCG may be used as a replacement for prime vaccine.

Additional new TB vaccine approaches include inactivated whole mycobacteria or fragmented mycobacteria (165). New TB vaccines of inactivated *M.vaccae* as well as detoxified cellular *M.tb* fragments have already been tested in clinical trials; both have showed good safety profile (171, 172). In a randomized, placebo-controlled, double-blind trial involving HIV infected adults with a history of childhood BCG vaccination, administration of multiple shots of *M.vaccae* was shown to confer significant protection against TB (172). RUTI, a novel vaccine comprised of detoxified and liposomed cellular fragments of *M.tb* was shown to be safe and immunogenic in a phase 1 clinical trial conducted with healthy adults (171). Other vaccine design approaches at the preclinical stage include attenuation of *M.tb* (64) and naked DNA vaccines encoding mycobacteria antigens such as Ag85A and Ag85B (173).

The global BCG vaccination coverage is over 80% (57) and the safety, effectiveness of BCG in preventing severe TB and other mycobacterial diseases in children is well established (125). Consequently, there are concerns that replacing BCG altogether may negatively interfere with its targeted and non-targeted benefits. It is therefore important to understand how BCG-induced immunity may be enhanced. Enhanced priming may result to optimal boosting and possibly increased effectiveness against TB. Studies in chapter five of this thesis evaluated whether a delay in BCG administration in infants may enhance the BCG-induced immunity.

In summary, the new TB vaccines under development might exert their effect in several ways: (i) prevent initial infection (ii) prevent primary TB disease (iii) prevent establishment of latency (iv) prevent reactivation of latent infection or (v) shorten the course of disease and improve the response to chemotherapy (52). A holistic view of the vaccination strategies and their associated immune mechanisms thought to confer protection are shown in **Figure 4**. **Table 1** provides a description of different types of novel TB vaccines reportedly tested in clinical trials.



**Figure 4:** Novel TB vaccine prime-boost vaccination strategies. TB vaccines may be preventative (to prevent *M.tb* infection), suppressive (suppress *M.tb* establishment after infection), prevent TB reactivation, or therapeutic. Vaccines may induce immune cells thought to be important in TB immunity, which include: (a) Th1 cells producing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, (b) Th17 cells producing IL-17, (c) Cytotoxic T lymphocytes (CTL) producing cytotoxic molecules. The Th2 cytokines and regulatory T cells (Tregs) may dampen the effector response. Another inhibitory mechanism of effector response is exhaustion of T cells (increased PD1 expression) due to persisting high *M.tb* loads as reviewed by Parida *et al.* (52).

Type of TB vaccine	Example	Description
Recombinant live	VPM 1002	rBCG prague strain expressing listeriolysin and carries a urease deletion mutation
	rBCG30	rBCG Tice strain expressing 30 kDa <i>M.tb</i> antigen 85B; phase I completed in U.S.
Viral vector vaccine	Oxford MVA85A / AERAS-485	Modified vaccinia Ankara vector expressing <i>M.tb</i> antigen 85A
	Crucell Ad35/ AERAS-402	Replication-deficient adenovirus 35 vector expressing <i>M.tb</i> antigens 85A, 85B, TB10.4
	AdAg85A	Replication-deficient adenovirus 5 vector expressing <i>M.tb</i> antigen 85A
Recombinant protein	Hybrid-I+IC-31	Adjuvanted recombinant protein composed of <i>M.tb</i> antigens 85B and ESAT-6
	Hybrid-I+CAF01	Adjuvanted recombinant protein composed of <i>M.tb</i> antigens 85B and ESAT-6
	M72	Recombinant protein composed of a fusion of <i>M.tb</i> antigens Rv1196 and Rv0125 & adjuvant
	HyVac 4/AERAS-404	Adjuvanted recombinant protein composed of a fusion of <i>M.tb</i> antigens 85B and TB10.4
Other	RUTI	Fragmented <i>M.tb</i> cells
	<i>M. vaccae</i>	Inactivated whole cell non-TB mycobacterium; phase III in BCG-primed HIV+ population completed; reformulation pending
	<i>M. smegmatis</i>	Whole cell extract; phase I completed in China

**Table 1:** Novel TB vaccine candidates in clinical trials in the year 2009. Stop TB Partnership website (174).

### 1.9 Assays to measure tuberculosis vaccine take

Bacillus Calmette-Guérin and novel TB candidate vaccines are aimed to mainly induce T cell immunity. Hence, immunological assays are designed to measure this type of vaccine-specific immunity. In humans, the majority of these assays evaluate the magnitude or quality of vaccine-specific memory T cells in the peripheral blood. Unlike lymph nodes, lung tissue and bronchial alveolar lavage, human peripheral blood is easier and less invasive to obtain hence suitable for these assays. Typically, most assays utilize *in vitro* restimulation of peripheral whole blood or peripheral blood mononuclear cells (PBMC) with vaccine antigens to mimic a secondary antigen encounter

(175). At protein level, the responding cytokine-producing cells can be measured by Elisa, Elispot and flow cytometry, for example (176), while at molecular level, methods such as quantitative real time polymerase chain reaction (PCR) are routinely used (176). The choice of method to use largely depends on the selected outcomes of interest and the resources available.

In TB vaccine clinical trials, whole blood or PBMC may be used when fresh, after cryopreservation or both. The duration of the immunological assay may also vary depending on practicalities and the nature of the question being addressed; (i) long-term assay (four or more days), (ii) medium-term assay (one to three days) or (iii) short-term assay (less than 24 hours) (175).

Long and medium term assays measure the capacity of antigen experienced cells to proliferate and acquire effector activity (177). Proliferating cells may also be phenotyped in these assays, although incubation may change surface phenotypes. These assays are thought to be ideal to measure a  $T_{cm}$  response, as these cells may require longer stimulation for activation. Expansion allows for an increase in sensitivity of the measured response. These assays are less affected by the time to incubation of blood or isolated PBMC with antigens, compared with short term assays. Finally, long and medium term assays are less affected by the manipulation at PBMC isolation and cryopreservation, compared with short-term assays (175). Long- and medium term assays are labour and resource intensive, and may be vulnerable to contamination during the culture period. Long and medium term assays are labour and resource intensive, and may be vulnerable to contamination during the culture period. These results are semi-quantitative, reflecting phenotype and function of expanded cells – these phenotypes may not have been the same at the onset of the assay (175). The selection of a suitable assay depends on what is practical within the study settings. In this thesis, we chose to utilize short-term whole-blood assays for several reasons. First, a “snapshot” of immunity present at the time of blood collection, in an entire immune compartment (peripheral blood) is measured. Second, very small blood volumes are required, which is an important consideration in pediatrics. Third, our laboratory has shown that immediate

stimulation of whole blood increases sensitivity of the measured T-cell response, compared with stimulation of cryopreserved PBMC. Fourth, assay backgrounds are lower than those of PBMC-based assays, further increasing the sensitivity (178).

Regardless of whether whole blood or PBMC is used, optimization of the chosen assay system is key in generating reliable immunological data. Resource permitting, performing a combination of short-, medium- and long-term assays would yield more information. We used a short-term whole blood assay followed by intracellular cytokine staining (ICS) analysis on a PFC. The short-term whole blood assay was optimized using whole blood from adults and 10-week old BCG vaccinated infants (178). Every stimulation step of the assay for this application was evaluated and optimized (178). This assay is robust and delivers specific, sensitive, and reproducible results (intra-assay coefficients of variation of less than 10%).

#### **1.10 Contributions**

B.M.N.Kagina wrote this chapter with supervision by Prof. W.A. Hanekom and Dr. B. Abel.

## **Chapter 2: Long-term cryopreservation of stimulated adult whole blood does not affect detection of IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells as measured by an intracellular cytokine assay**

Results presented in this thesis were derived from examination of stimulated, fixed cells, obtained from whole blood incubated with specific antigens, and cryopreserved for long periods. Our first objective was to assess whether long-term cryopreservation would allow reliable measurement of mycobacteria induced immune responses.

### **2.1 Background**

Our laboratory is involved in assessing immunity induced by novel TB vaccines and investigating immune correlates of risk/protection against TB. To date, thousands of infant, adolescent, and adult whole blood and PBMCs have been collected, processed and cryopreserved for analysis (179). Cryopreservation of cells is a common practice in clinical immunological studies that incorporate long follow-up periods, or which involve large sample sizes. In these settings, it is often impractical to complete assays in real time; moreover, in longitudinal studies, batch analysis of samples from individual participants is often preferred because of a reduction in variability of assay conditions. In addition, batch analysis is often more cost effective.

Cryopreservation may be defined as the maintenance of biological samples in cold storage. Cells are typically stored at temperatures below  $-140^{\circ}\text{C}$ . Mononuclear cells may be cryopreserved prior to stimulation; alternatively, these cells or whole blood may be incubated with specific antigens prior to cryopreservation. Cryopreservation of viable cells in specific media at very low temperatures may enable long-term preservation of many functional properties of the cells (180).

The cryopreservation process begins with a stepwise reduction in temperatures (181). As the cells cool to temperatures between  $-5$  to  $-15^{\circ}\text{C}$ , ice forms (181). Extracellular ice formation creates an osmotic gradient

across the cell membrane that causes osmotic stress, resulting in water being drawn out of the cells (180). Further reduction of temperatures leads to increased extracellular ice crystal formation, which can result in cellular injury, cell membrane shrinking, and ultimately a toxic increase in the concentration of intracellular solutes (180). The faster the cooling rate, the smaller the ice crystal formation and vice-versa (180). Very rapid cooling can minimize ice crystal formation almost completely resulting in vitrification of the solutes and increased cell viability (180); however, in practice, this is not fully achievable without cryoprotectants. The addition of cryoprotectants prior to cooling increases viscosity of the extracellular solution (180). Consequently, freezing point and ice crystal formation of the solution are lowered allowing vitrification temperatures to rise during the freezing process (180). This minimizes cellular injury during cryopreservation. Extensive progress has been made in the field of cryoprotectants (182). Dimethyl sulfoxide (DMSO), glycerol and propylene glycol are the most commonly used cryoprotectant agents (180).

For immunology studies, cryopreservation may be of viable PBMCs or of fixed cells from stimulated whole blood. The advantages of cryopreserving viable PBMCs include the following: multiple vials of cells may be frozen from the same donor allowing for repeat analysis, stimulation antigens can be conveniently introduced post-thawing, and viability markers are easily incorporated when performing functional assays. The disadvantages of cryopreserving PBMC include: large volumes of blood are required, viability may be compromised during the thawing process, and activation of caspases may occur leading to reduced cellular function (180, 182).

Cryopreservation of fixed cells from stimulated whole blood is common in clinical trials with immunology components. Fresh whole blood is first incubated with selected antigens for a predefined period. Then, brefeldin A or monensin may be added to trap secreted cytokines intracellularly (183, 184). Red blood cells are then lysed and white blood cells harvested. Finally, the white blood cells are fixed with fixative agents followed by cryopreservation. Commonly used fixative agents include glutaraldehyde and



paraformaldehyde (185). Fixative agents cause cross-linkage of cell membrane components allowing preservation of cell membrane integrity (186). This blocks penetration of high molecular weight compounds into the cell during cryopreservation. Assays performed with cryopreserved fixed cells from stimulated whole blood require smaller blood volumes, less resources (during stimulation steps), and are generally more sensitive than viable PBMC samples stimulated post-cryopreservation. A disadvantage of cryopreserving fixed cells from stimulated whole blood is that stimulations cannot be conducted retrospectively, which prevents flexibility later in the assay.

Our aim in this chapter was to assess whether long-term cryopreservation of stimulated and fixed white cells affect the mycobacteria-induced immune response when measured by ICS assay. We hypothesized that BCG-, PPD- and SEB-induced CD4 T cell IFN- $\gamma$  response is not reduced when fixed cells obtained from whole blood incubated with specific antigens is cryopreserved for long periods. We also evaluated the effects of prolonged cryopreservation on PBMC viability.

## **2.2 Materials and methods**

### **2.2.1 Study participants and blood collection**

Healthy adults were recruited at the Institute for Infectious Diseases and Molecular Medicine of the University of Cape Town in South Africa. All participants had received BCG at birth and lived in the Western Cape Province of South Africa, a region with a very high incidence of TB disease (187). Study participation by all volunteers was approved by the University of Cape Town research ethics committee using the guidelines of the National Health Research Ethics Council. Good clinical practice guidelines were adhered to and this included written informed consent from the participants. For the short-term whole blood assay, 200mL blood was collected from each donor in heparinized tubes. For the PBMC isolation, 200mL blood was collected from each donor in cell preparation tubes (CPT). Donors were

either bled for the short-term whole blood assay or PBMC isolation, but not both.

### **2.2.2 Antigens**

BCG Danish 1331 (Statens Serum Institut, Copenhagen) was prepared from the vaccine vial by reconstitution with RPMI and used at a final concentration of  $1.2 \times 10^6$  CFU/mL of blood, a dose found optimal in preliminary experiments (178). Purified protein derivative (PPD, Statens Serum Institut, Copenhagen) was used at a final concentration of 10µg/mL and Styphylococcal enterotoxin B (SEB, Sigma), a positive control, was used at a final concentration of 10µg/mL as previously described (178).

### **2.2.3 Short-term whole blood stimulation and cryopreservation**

Tubes containing one mL whole blood volumes from each participant were either left unstimulated (negative control) or stimulated with BCG, PPD or SEB (positive control). The co-stimulatory antibodies anti-CD28 and anti-CD49d (both from BD Biosciences, 1µg/mL) were included in all assay conditions. These co-stimulatory antibodies have been shown to enhance the specific cytokine response (178). The whole blood was incubated at 37°C for 12 hours, and Brefeldin-A (Sigma, 10µg/mL) was added for the last five hours of incubation. The blood was then harvested with EDTA (Sigma, 2µM), red blood cells lysed and white cells fixed with FACS lysing solution (BD Biosciences). Later, white cells were pelleted and multiple aliquots per antigen/ condition per donor were cryopreserved in 10% DMSO (Sigma) in fetal calf serum (BioWest).

### **2.2.4 Intracellular cytokine staining**

One week after phlebotomy, an aliquot of cryopreserved fixed cells from whole blood incubated with each of nil, BCG, PPD, and SEB was thawed at 37°C, cells were washed, permeabilized with perm/wash solution (BD Biosciences) and incubated for one hour with fluorescence-conjugated antibodies against intracellular IFN-γ and surface antigen CD4. The fluorescence-conjugated antibodies used were CD4-APC (SK3) and IFN-γ-

PE (25723.11), both from BD Biosciences. Flow cytometric acquisition was performed on a FACSCalibur (BD Biosciences) and at least 40,000 events were acquired. Subsequently, aliquots of the cryopreserved fixed cells were thawed and similarly processed at 3 monthly intervals. Flow analysis was performed using Flowjo software (9.0.2, TreeStar).

### **2.2.5 PBMC isolation and cryopreservation**

PBMC were isolated using CPT (BD Biosciences). Immediately after isolation, viable recovery was assessed using trypan blue (Sigma) exclusion staining. For each participant, multiple cryovials containing  $10^7$  viable PBMC in 1mL 10% DMSO in fetal calf serum were step-frozen: vials were placed overnight in “Mr. Frosty” containers (Nalgene/Merck) containing isopropyl alcohol to allow step-wise freezing to  $-80^{\circ}\text{C}$ . The following morning, the vials were transferred into liquid nitrogen for long-term storage.

### **2.2.6 PBMC thawing and viability**

At three month intervals and over a period of four years, cryopreserved PBMC were rapidly thawed at  $37^{\circ}\text{C}$ . When only a small ice pellet remained, RPMI containing 5% human AB serum (Western Province blood Transfusion, SA), (BioWhittaker) was added dropwise, with gentle shaking of the cryovial. The amount of medium added was doubled after each shaking. When the cryovial was full, the contents were transferred into a 15mL conical polypropylene tube (BD Biosciences), and medium was added dropwise to fill the tube. PBMC were washed twice before resuspension in medium, and viability determined by use of trypan blue exclusion stain.

### **2.2.7 Data analysis**

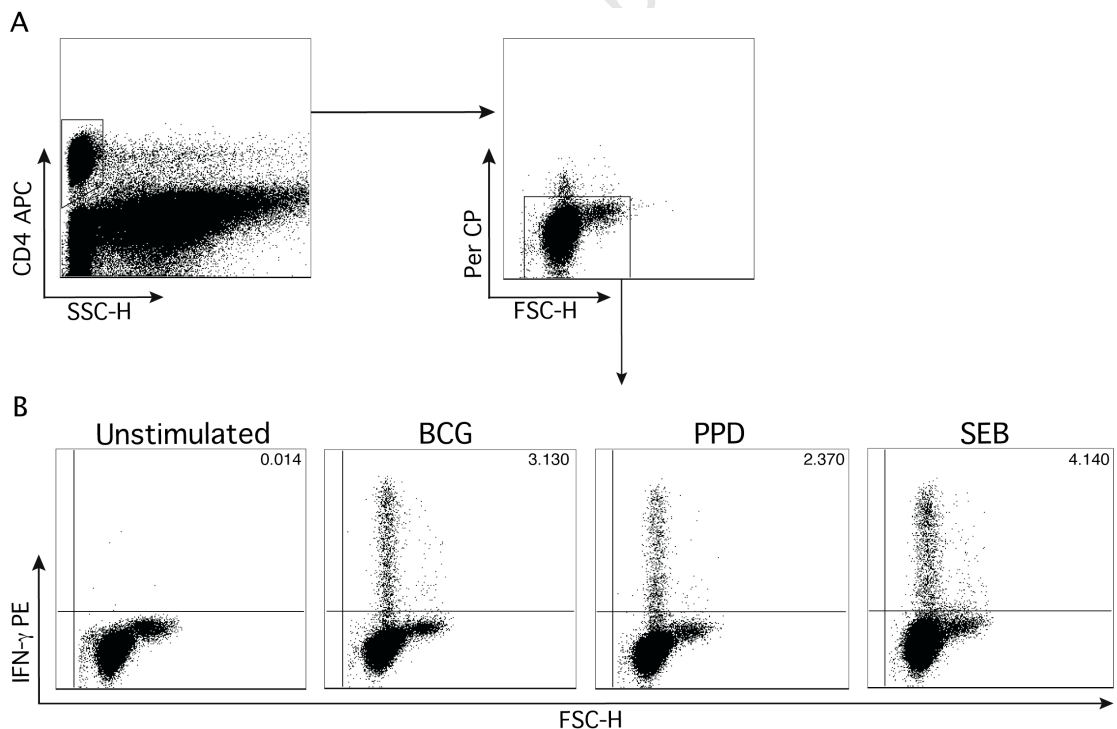
The frequencies of  $\text{IFN-}\gamma^+ \text{CD4}^+$  T cells over time, in the nil, BCG, PPD and SEB conditions were assessed for all the donors. The unstimulated values were subtracted from the BCG, PPD and SEB values. The coefficient of variation (CV) analysis was used to measure the variability in the median frequencies of  $\text{IFN-}\gamma^+ \text{CD4}^+$  T cells over time. The viable PBMCs were expressed as a percentage of the total cells (live and dead). Over time, the

CV analysis was used to measure the variability in the frequencies of viable PBMC for all the donors. The one way analysis of variance (ANOVA) test was used to assess whether the mean viable PBMC for all the donors over time were significantly different. P values less than 0.05 were considered significant. All the data was analysed using GraphPad Prism version 5.0a.

## 2.3 Results

### 2.3.1 Gating strategy for the whole blood intracellular assay

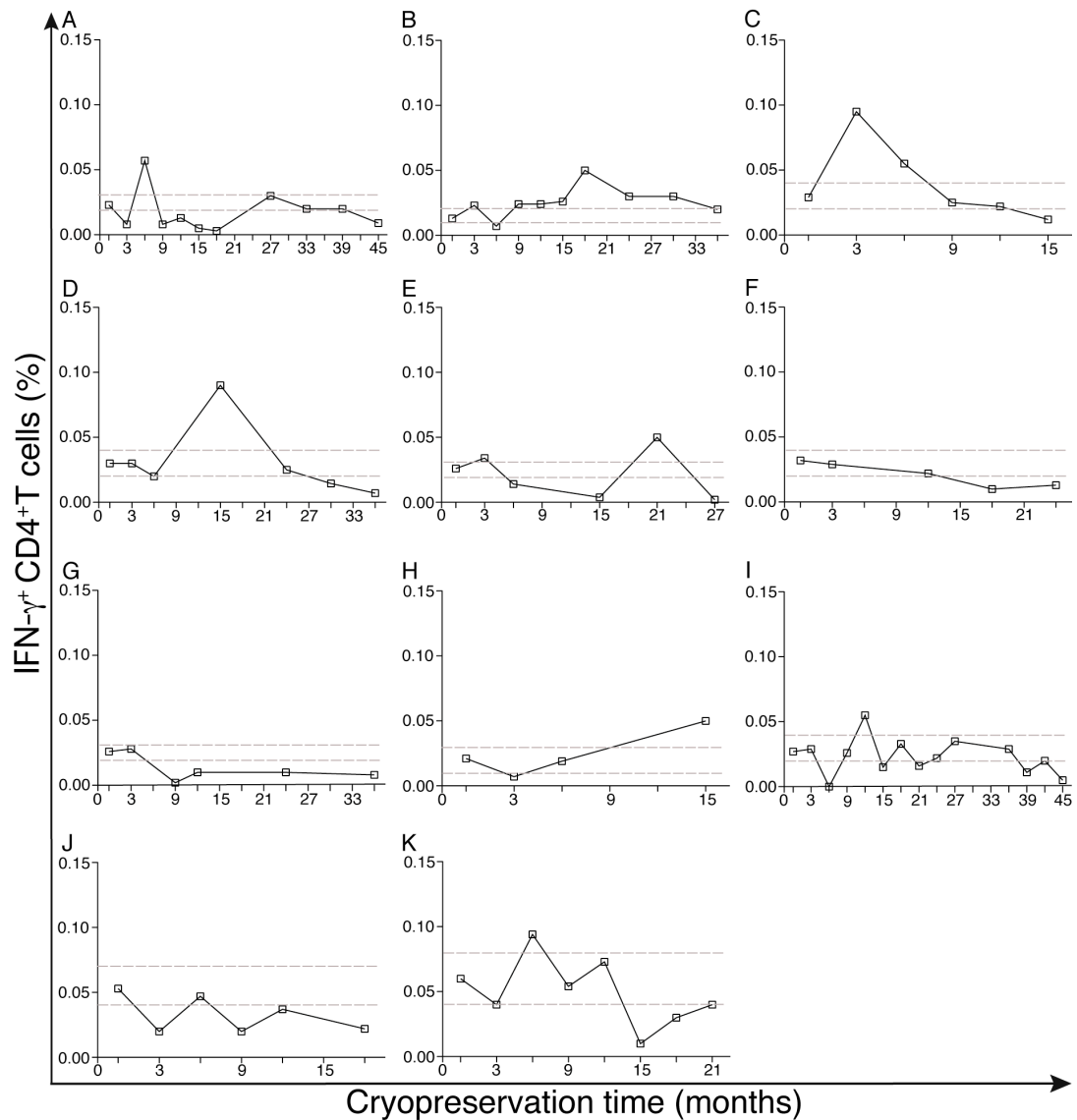
We used the gating strategy shown in **Figure 5** to measure the unstimulated and the mycobacteria-specific responses following long-term cryopreservation. This strategy enables exclusion of background autofluorescence and doublet cells, which would otherwise reduce the reliability of the results.



**Figure 5: Gating strategy.** CD4<sup>+</sup> T cells were first selected by gating CD4 against side scatter (SSC). We then used an exclusion gate comprising Forward Scatter (FSC) against the empty PerCP channel (**A**). An example of flow cytometric detection of IFN- $\gamma$  expressing CD4<sup>+</sup> T cells when whole blood was left unstimulated or following incubation with BCG, PPD or SEB, and costimulants (unstimulated), for 12 hours. Representative dot plots of IFN- $\gamma$  expression in CD4<sup>+</sup> T cells from unstimulated, BCG, PPD and SEB-stimulated conditions from a single donor after cryopreservation of stimulated whole blood for 36 months (**B**).

### **2.3.2 IFN- $\gamma$ <sup>+</sup> expression in CD4<sup>+</sup> T cells from unstimulated blood does not increase following long-term cryopreservation**

In our laboratory, we have observed increased expression of intracellular cytokines in unstimulated PBMC that were frozen, thawed and incubated, compared with stimulation of PBMC immediately after isolation. In the whole blood assay, cells are fixed after stimulation, and later thawed for flow cytometric analysis. We wished to assess whether similar increases in unstimulated IFN- $\gamma$ <sup>+</sup> expression would be observed with the whole blood assay after cryopreservation. This IFN- $\gamma$ <sup>+</sup> expression was assessed one week after cryopreservation, and 3 monthly thereafter, up to 45 months later. We showed that in the majority of participants, background frequencies of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells remained within a narrow margin, and did not increase over time (**Figure 6A-K**). We concluded that cryopreservation did not result in increased unstimulated expression of IFN- $\gamma$ .



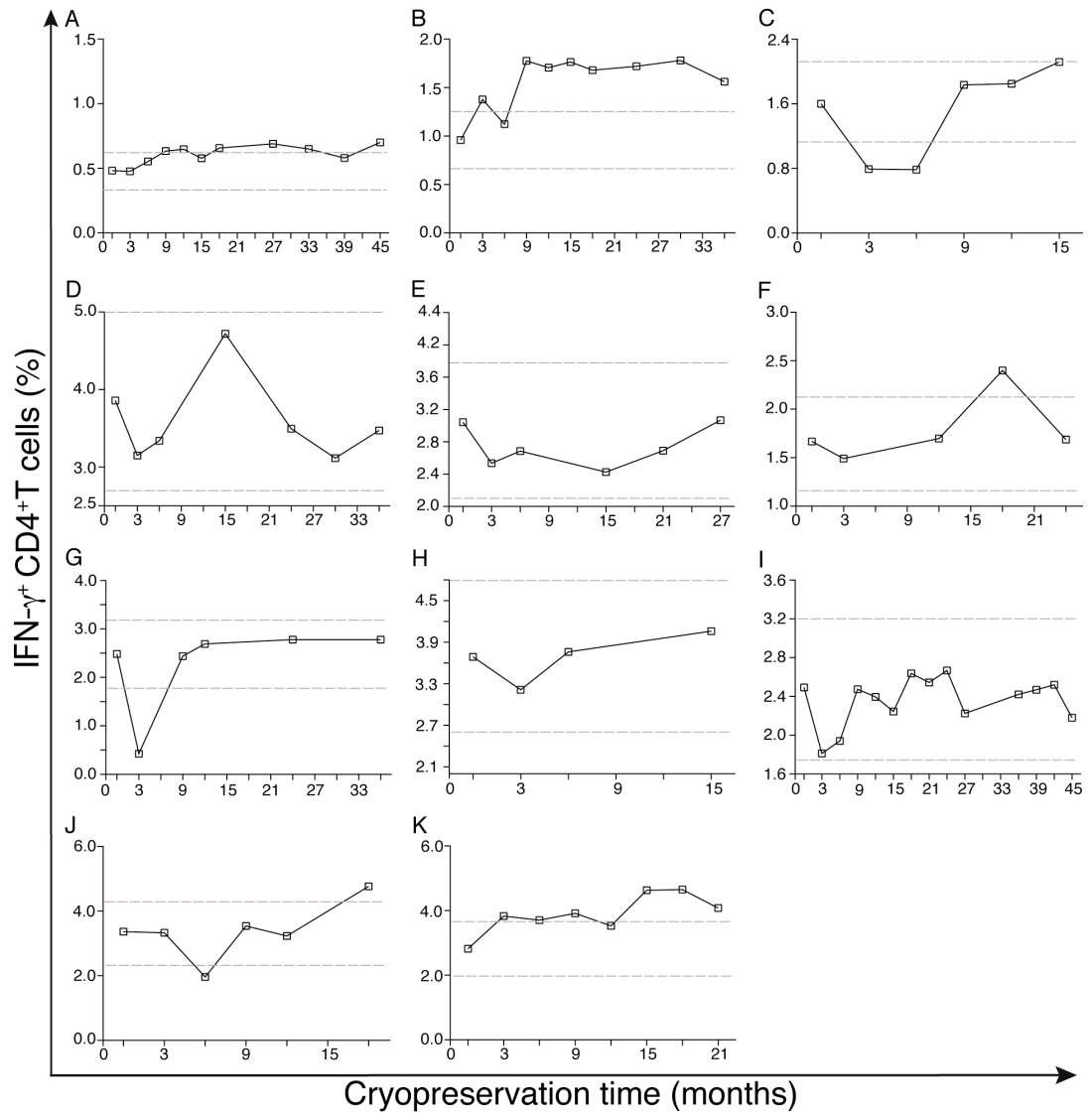
**Figure 6: Longitudinal background frequencies of IFN- $\gamma^+$  in CD4 $^+$  T cells.** For the 11 donors analyzed (A-K), we utilized the gating strategy described in Figure 1 to measure the background (unstimulated) frequencies of IFN- $\gamma^+$ -expressing CD4 T cells from 1 week after cryopreservation, and 3 monthly thereafter, up to 45 months later. Samples from the majority of donors were analyzed at all time points, but only up to 18 months. Only 5 donors had their samples analyzed beyond the 3-year period. The dashed lines indicate values corresponding to 30% variation relative to the frequency of IFN- $\gamma^+$  CD4 $^+$  T cells observed at one week after cryopreservation.

### 2.3.3 No change in the frequency of mycobacteria-specific IFN- $\gamma^+$ -expressing CD4 $^+$ T cells following long-term cryopreservation of stimulated whole blood

Next, we assessed whether frequencies of mycobacteria-specific IFN- $\gamma^+$  CD4 $^+$  T cells changed following long-term cryopreservation of whole blood stimulated with either viable BCG or PPD, respectively. This IFN- $\gamma^+$

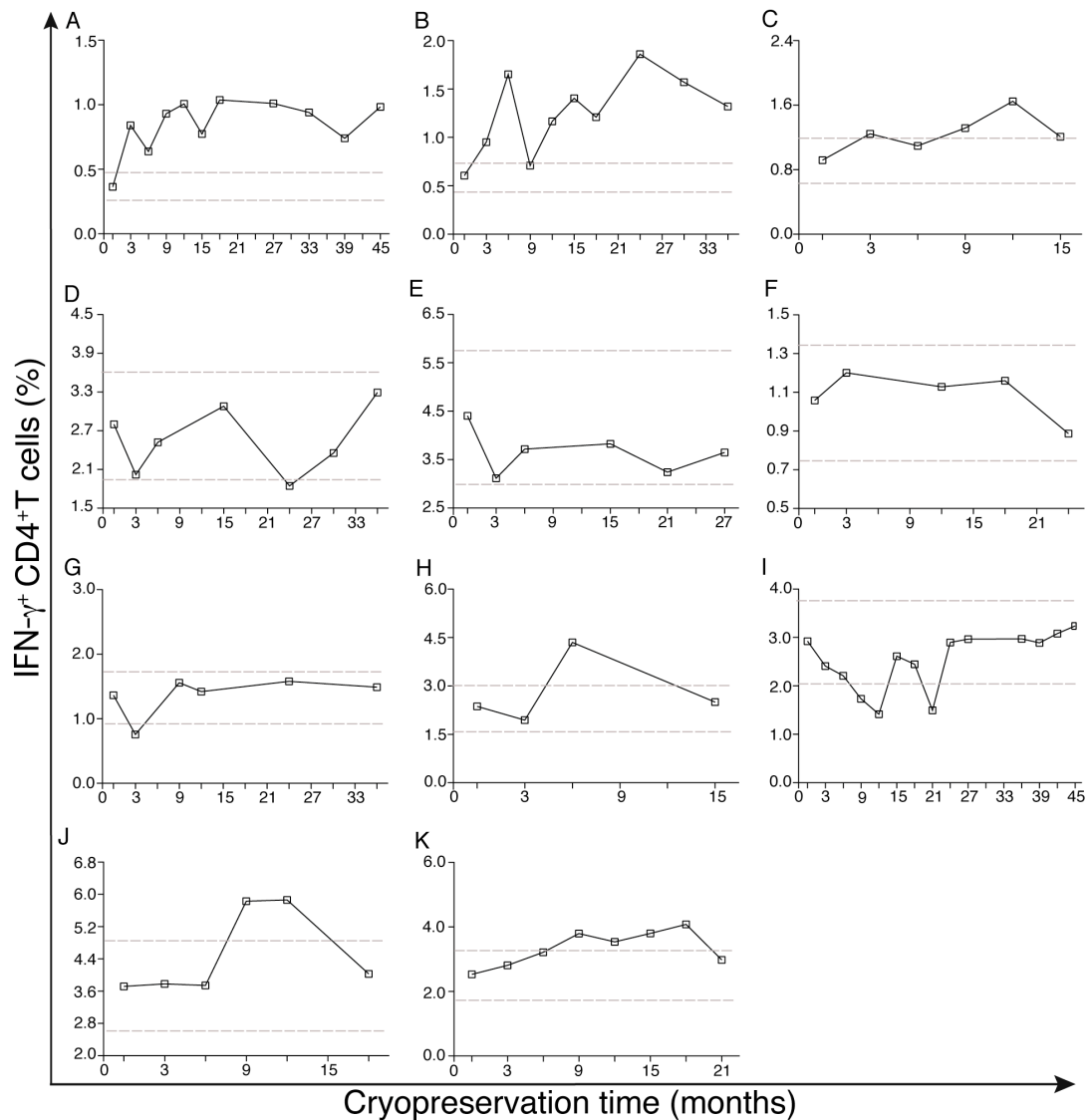
expression was assessed one week after cryopreservation, and 3 monthly thereafter, up to 45 months later. The frequencies of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells remained within a very narrow range (**Figures 7A-K and 8A-K**). We concluded that cryopreservation did not result in a change in the frequencies of mycobacteria-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup>T cells.

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**Figure 7: Longitudinal BCG-specific frequencies of IFN- $\gamma$  in CD4<sup>+</sup> T cells.** For the 11 donors analyzed (A-K), we utilized the gating strategy described in Figure 1 to measure the BCG-specific frequencies of IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells. Background (unstimulated) frequencies were subtracted from the respective BCG samples for the relevant time point analyzed. Analysis was performed from 1 week after cryopreservation, and 3 monthly thereafter, up to 45 months later. Samples from the majority of donors were analyzed at all time points, but only up to 18 months. Only 5 donors had their samples analyzed beyond the 3-year period. The dashed lines indicate values corresponding to 30% variation relative to the frequency of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells observed at one week after cryopreservation.



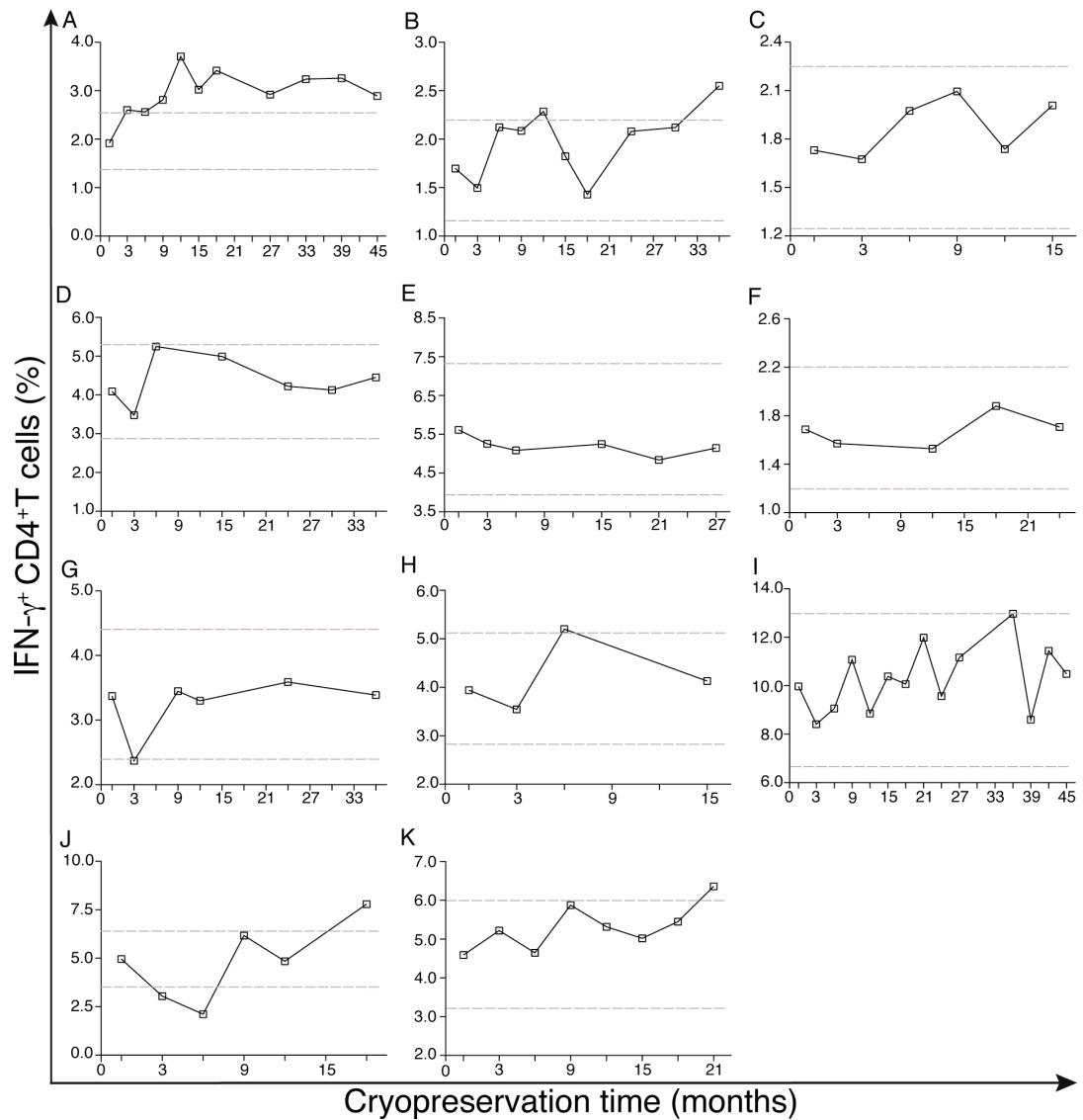


**Figure 8: Longitudinal PPD-specific frequencies of IFN- $\gamma$  in CD4<sup>+</sup> T cells.** For the 11 donors analyzed (A-K), we utilized the gating strategy described in Figure 1 to measure the PPD-specific frequencies of IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells. Background (unstimulated) frequencies were subtracted from the respective PPD samples for the relevant time point analyzed. Analysis was performed from 1 week after cryopreservation, and 3 monthly thereafter, up to 45 months later. Samples from the majority of donors were analyzed at all time points, but only up to 18 months. Only 5 donors had their samples analyzed beyond the 3-year period. The dashed lines indicate values corresponding to 30% variation relative to the frequency of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells observed at one week after cryopreservation.

### 2.3.4 No change in the frequency of SEB-induced IFN- $\gamma$ -expressing CD4<sup>+</sup> T cells following long-term cryopreservation of stimulated whole blood

In intracellular cytokine staining assays, a positive control is critical for ascertaining whether the assay was successful – if this control yields

suboptimal responses, results from other conditions should not be included in data analysis. We assessed whether long-term cryopreservation would result in changes in IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells after SEB stimulation of whole blood. IFN- $\gamma$ <sup>+</sup> expression was assessed one week after cryopreservation, and 3 monthly thereafter, up to 45 months later. We showed the frequency of SEB-induced IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells did not change significantly (**Figures 9A-K**). We concluded that there was no change in the frequencies of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells after SEB stimulation followed by longterm cryopreservation. However all donors displayed levels that fluctuated during the period studied, which is likely due to the ICS assay variability.



**Figure 9: Longitudinal frequencies of IFN- $\gamma$  in CD4<sup>+</sup> T cells in SEB stimulated whole blood samples.** For the 11 donors analyzed (A-K), we utilized the gating strategy described in Figure 1 to measure the frequencies of IFN- $\gamma$ <sup>+</sup>-expressing CD4<sup>+</sup> T cells following SEB stimulation of whole blood. Background (unstimulated) frequencies were subtracted from the respective SEB stimulated samples for the relevant time point analyzed. Analysis was performed from 1 week after cryopreservation, and 3 monthly thereafter, up to 45 months later. Samples from the majority of donors were analyzed at all time points, but only up to 18 months. Only 5 donors had their samples analyzed beyond the 3-year period. The dashed lines indicate values corresponding to 30% variation relative to the frequency of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells observed at one week after cryopreservation.

### 2.3.5 Frequencies of mycobacteria-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells vary marginally following long-term cryopreservation

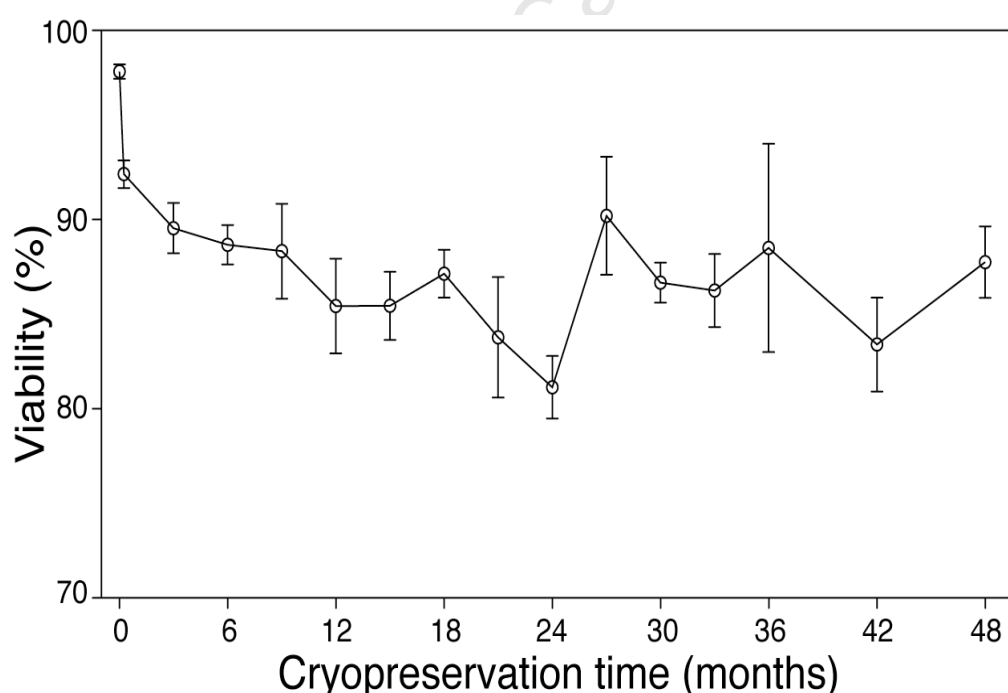
To assess whether frequencies of unstimulated, mycobacteria-specific, and SEB-induced IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells are compromised following long-term cryopreservation of fixed white cells from stimulated whole blood, we evaluated the intra-donor variability of the unstimulated, BCG-, PPD-, and SEB-induced responses measured at different time points (median of 6 time points). The median CVs were 73.7, 15.5, 20.9, and 13.1% for unstimulated, BCG, PPD and SEB conditions, respectively (**Table 2**). We concluded that the observed variations in the frequencies of mycobacteria-specific and SEB-induced IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells were within acceptable ranges following long term cryopreservation.

Donor ID	Number of time points analyzed	Coefficient of variation (%)			
		Uns	BCG	PPD	SEB
A	11	94.6	8.5	16.9	11.8
B	10	46.8	19.1	32.5	17.9
C	6	77.3	38.3	19.7	9.4
D	7	88.4	15.5	20.9	13.6
E	6	86.1	9.6	12.6	4.9
F	5	45.0	19.7	11.4	8.2
G	6	73.7	40.3	22.6	13.5
H	4	75.2	9.5	38.2	16.9
I	14	59.9	10.6	23.8	13.1
J	6	44.1	26.5	23.5	42.7
K	8	52.1	15.3	16.3	11.2
Median	6	73.7	15.5	20.9	13.1

**Table 2: Intra-donor coefficient of variation of the assay.** Results are shown for intra-donor CVs for frequencies of Unstimulated (Uns), BCG-, PPD-, and SEB-induced IFN- $\gamma$ -expressing CD4<sup>+</sup> T cells for all the time points analyzed. Unstimulated responses were subtracted from the respective stimulated responses for each time point. The number of time points analyzed for individual donors are indicated. The minimum and maximum time points analyzed during cryopreservation were 4 and 14 respectively.

### 2.3.6 Peripheral blood mononuclear cells viability is significantly reduced within the first week of cryopreservation, but appears stable during long-term cyostorage

To assess the effects of long-term cryopreservation on the viability of PBMC, we assessed recovery of viable PBMC thawed 1 week after cryopreservation, and 3 monthly thereafter, for a period up to 48 months. We showed that the greatest reduction in viability occurred immediately after cryopreservation ( $p < 0.0001$ ), while the number of viable cells recovered at later time points remained remarkably similar ( $p > 0.05$  when any 2 time points were compared after 1 week. **Figure 10**). The average viability during the cryopreservation period was  $>80\%$ , a threshold level of  $70\%$  has been reported to be sufficient to perform optimum functional immunological assays (188). We concluded that long-term cryopreservation of viable PBMC is feasible in our setting.



**Figure 10: Longitudinal recovery of viable PBMC after cryopreservation.** Viable PBMC recovery rate after thawing cryopreserved cells one week after cryopreservation, and at 3-monthly intervals thereafter. Viability was determined by trypan blue exclusion. Mean and standard errors are shown at the indicated time points.

## 2.4 Discussion

We report that the frequencies of change of IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells detected by ICS assay are not compromised after long-term cryopreservation of stimulated, fixed cells, obtained from whole blood. We observed a significant reduction in the viability of PBMC after one week of cryopreservation, however at later time points, PBMC viability was maintained for up to 48 months after cryopreservation.

In biological assays, the CV is a measure of precision within or between repeated measurement values (188). In ICS assays, CVs of up to 30% may be considered acceptable (189). We observed CVs of <30% in the frequencies of antigen-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells. CVs of up to 23.6% have been previously reported in ICS assays using freshly processed and stimulated whole blood samples (189). It is therefore possible that the slightly higher CVs observed in our study may be due to ICS assay variability and not long-term cryopreservation. Batch variation in reagents used during the extensive period of the study (45 months), and variability introduced by multiple operators may also partly explain the observed CVs. The CVs for unstimulated samples were high (73.7%), which could be explained by the fact that the mean for IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells was near zero, thus rendering the CV extremely sensitive to small changes. In these experiments, we chose to evaluate changes in the frequency of mycobacteria-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells because these cells are the most highly induced in our assay system. It is rational to assume that the variation or stability in this outcome could be valid to other cytokines. Therefore, repeatability in measurements of low magnitude frequencies with ICS may be unreliable.

Our laboratory is highly experienced in flow cytometry analysis of mycobacteria-specific cytokine-expressing T cells, following long-term cryopreservation of stimulated, fixed cells, obtained from whole blood. We have observed a notable increase in the autofluorescence of cells, which could be associated with long-term cryopreservation (unpublished observations from our lab). In this study, we utilized an exclusion channel to

minimize the detection of auto-fluorescing dead cells, which may affect the reliability of the results. Currently, there are viability cell dyes that can be used to stain whole blood before cryopreservation (190). Use of such viability dyes may exclude the dead cells and ultimately minimize autofluorescence and false-positive signals in the channels of interest.

We observed stable frequencies of antigen-specific IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells in stimulated, cryopreserved fixed whole blood cells. The recovery of viable PBMC was significantly lower after one week of cryopreservation compared with all later time points. This indicated that ICS assays utilizing cryopreserved fixed cells from stimulated whole blood may be more suitable for our studies than cryopreserved PBMC. However, the decline in recoverable viable PBMC stabilized, and at least 80% viability was retained up to 48 months after cryopreservation. It is likely that the significant reduction in viability occurred during the overnight step freezing process and not later during the cryopreservation. Introduction and removal of cryoprotectants are critical steps in the maintenance of viable cells (191). High PBMC viability is a key variable in the performance of immunological assays. When using cryopreserved PBMC stimulated with either cytomegalovirus (CMV) or pokeweed mitogen (PWM), Weinberg *et al.* reported that a minimum viability of 70% was optimal for functional immunological assays (188); PBMC samples with viable recovery of less than 70% showed inconsistent proliferative responses. Although different functional assays may have variable PBMC viability requirement for optimal performance, it is widely known that high viability is critical in assays utilizing fresh or cryopreserved PBMC. Knowledge on how long-term cryopreservation of biological samples may affect immunological outcomes remains inadequate. A similar threshold of 70% in PBMC viability was reported to be critical for optimal proliferative T cell assays when cryopreserved PBMC were stimulated with tetanus toxoid (191). Incorporation of steps such as overnight resting of PBMC after thawing, and the use of a viability dye during analysis, greatly improves the functional capacity of T cells and minimizes detection of autofluorescence. This is the setting where the viability dye may be particularly useful.

Anecdotal experience in some vaccine studies conducted in SATVI show that stimulation of viable PBMC after cryopreservation may result in reduced cytokine expression levels when measured in ICS assays. Furthermore, Costantini *et al.* reported that cryopreservation of PBMC derived from healthy adults resulted in reduced surface expression of CD62L on T cells, and this was accompanied by decreased proportions of naive and central memory T cell subsets while proportions of effector T cells was increased (192). The same study reported that CCR5 expression on T cells was reduced when PBMC from HIV-infected adults were cryopreserved and this was associated with reduced T cell proliferative capacity after stimulation with p24, an HIV derived antigen, CMV and Influenza virus (192). Such findings are not surprising because during cryopreservation, cell death can occur due to increased cellular stress (182) and temperature fluctuations may cause mechanical stress and cellular damage (182). Therefore, in addition to optimizing the cryostorage and thawing processes, maintenance of the cryofacility and stringent control of the cryostorage conditions is critical. To assess the effects of long-term cryopreservation on mycobacteria-specific T cells, stable conditions were observed during cryopreservation via a rigorous monitoring system implemented by our laboratory. With this monitoring system, fluctuations in cryotank temperatures and levels of liquid nitrogen in these tanks are monitored continuously. Corrective measures are in place to ensure that fluctuations do not compromise the integrity of the frozen samples. All the samples used for this thesis were stored in our cryofacility.

In the period of 2002/3, SATVI collected stimulated whole blood and PBMC from more than 5,700 infants to determine correlates of risk of childhood TB disease development, following newborn BCG vaccination. All samples were cryopreserved in liquid nitrogen biobanks. Currently, several different studies are utilizing these samples to perform intracellular cytokine staining (ICS), proliferation and cytotoxicity assays. We conclude that implementation of optimized cryopreservation and thawing processes enables long-term cryostorage of stimulated whole blood and freshly isolated PBMC for optimum functional T cell analysis.



## **2.5 Contributions**

Study design, sample collection and laboratory analysis was done by Prof. W.A. Hanekom and other members of SATVI laboratory. B.M.N. Kagina conducted data analysis and wrote this chapter under supervision of Prof. W.A. Hanekom and Dr. B. Abel.

University of Cape Town

## **Chapter 3: Optimization of an 8-colour flow cytometric intracellular cytokine-staining assay to characterize BCG-specific T cells in infants**

### **3.1 Introduction**

In 2002/3, we designed a study to identify correlates of risk of childhood TB disease, following newborn BCG vaccination (Chapter four). This chapter focuses on the optimization of an 8-colour flow cytometric ICS panel to analyze the samples generated from this study.

Flow cytometry is a widely applied technique of analyzing fluorescently labelled cells in fluidics channels (193). Both phenotype and functional characteristics of cells may be measured (193). Flow cytometers have evolved from three parameters, used in the late 1960s to more advanced polychromatic cytometers that could measure six parameters per cell in the 1980s (194). In the 1990s, hardware, software and chemistry developments facilitated further technological advancements on polychromatic cytometers to measure more than ten parameters (194). Today, cytometers that can measure up to 20 parameters are available, allowing more complete and complex immunological analyses (193). Simultaneous analysis of a large number of parameters in a single cell using the advanced cytometers is commonly referred to as polychromatic flow cytometry (PFC).

The advantages of PFC are multiple. For example, profiles of distinct antigen-specific T cell responses induced by a vaccine can be identified, based on functional (e.g cytokine) and phenotypic (e.g memory subsets) characteristics. Additionally, in pediatric study settings where limited volumes of blood samples are available, the use of PFC allows a researcher to obtain maximum information from small sample volumes. However, the benefits afforded by PFC are only achieved after meticulous optimization steps, more so when large polychromatic panels are utilized (195). These steps include: (i) selection of combinations of phenotypic and functional markers that may optimally be used together, (ii) optimal configuration of the flow cytometer, (iii) selection of optimal antibody-fluorochrome combinations, (iv) determining the optimal photomultiplier tube (PMT) voltages, (v) establishing the flow

cytometer's lower limit of detection, (vi) assessing spectral overlap by fluorescent minus one (FMO) controls, (vii) determining the intra-assay CV, and (viii) development of optimal gating strategy.

This chapter describes how the optimization steps were rolled out to develop and optimize an 8-colour PFC panel. First, we describe how we utilized existing information to guide us in the design of optimization experiments. Second, in the results section of this chapter, we describe experiments that addressed the aforementioned technical challenges. The optimized PFC panel was used for studies described in chapter four and minor modifications of the panel were implemented for studies described in chapter five.

### **3.1.1 Selection of phenotypic and functional markers**

Our broad aim is to identify correlates of risk of childhood TB disease, following newborn BCG vaccination. Our knowledge of immune correlates of protection against TB is inadequate. The following is a rationale for considering inclusion of the following 10 markers in the panel: CD3, CD4, CD8, gamma delta ( $\gamma\delta$ )T, IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-13, IL-17 and IL-22.

We know that CD4 and CD8 T cell immunity is important for controlling progression of *M.tb* infection to TB disease (196, 197), and this kind of immunity is therefore the most commonly measured when evaluating mycobacterial immunity, and when assessing the immunogenicity of novel TB vaccines (105, 106). We also know that BCG mainly induces a Th1 response (130).

We considered measuring  $\gamma\delta$  T cells for the following reasons. In a clinical setting, it has been shown that PBMC derived from BCG vaccinated adults have a higher  $\gamma\delta$  T cell response compared with PBMC derived from BCG unvaccinated controls, upon *in vitro* restimulation with *M.tb* whole lysate, with BCG, or with the phosphoantigen isoprenyl pyrophosphate (IPP) (198).  $\gamma\delta$  T cells have been reported to express more IFN- $\gamma$  in swine newborns vaccinated with BCG compared with unvaccinated controls (199). In a clinical

setting, it has also been shown that  $\gamma\delta$  T cells and immature DC can bi-directionally activate each other via CD1c surface molecule interaction, resulting in maturation of the DCs (200). The same study reported that the presence of both LPS and CD1-restricted  $\gamma\delta$  T cells was important to drive maturation of DCs with concomitant production of IL-12 and increased differentiation of naive T cells to Th1 cells (200). This observation implies an indirect association between  $\gamma\delta$  T cells and the induction of anti-mycobacterial CD4 and CD8 T cells.

As regards selection of cytokines, evidence suggesting increased incidence and severity of mycobacterial disease in human immune deficiencies from IFN- $\gamma$ RI+II-, IL-12R $\beta$ 1-, IL-12p40-, Stat1 (201-203) or acquired HIV infection (204, 205), have emphasized the critical role of conventional Th1 cells producing IFN- $\gamma$ . There is data supporting the role of IL-2 in the maintenance and differentiation of T and NK cells (206, 207). These two subsets (T and NK cells) have crucial roles in TB immunity (197, 208). TNF- $\alpha$  has been reported to play a protective role in TB immunity. Observations that patients with rheumatoid arthritis and treated with anti-TNF- $\alpha$  monoclonal antibody were at increased risk of TB reactivation highlighted the crucial role of this pro-inflammatory cytokine (209). TNF- $\alpha$  knock out mice are not only more susceptible to *M.tb* infection and development of TB than wild type mice but also have poorly formed granuloma structures in the lungs (210). This suggests a possible role of TNF- $\alpha$  in the formation and maintenance of granuloma, structures presumed to help contain *M.tb* infection within the host. We postulated that BCG-induced Th1 immunity would correlate with reduced risk of TB disease development. In a short-term whole blood assay, our laboratory has shown that BCG-specific Th1 cells are readily measureable in 10-week old infants, routinely vaccinated with BCG at birth (130). We therefore included IFN- $\gamma$ , IL-2 and TNF- $\alpha$  in our panel.

In clinical and experimental studies, it has been shown that Th2 cells producing IL-4, IL-5 and IL-13, may dampen presumably protective vaccine-specific Th1 cell responses (211). We therefore considered evaluating BCG-

specific Th2 cells. However, using a similar assay system, our group previously demonstrated that the T cell intracellular expression levels of IL-4 and IL-5 were too low to allow for reliable analysis in BCG vaccinated 10-week old infants (130). Thus, we considered including IL-13 in the panel, but not the other 2 cytokines (see below).

Studies in our laboratory have shown mycobacterial-specific IL-17 and IL-22 cytokine producing CD4 T cells in peripheral blood of healthy adults exposed to mycobacteria (212). Both IL-17 and IL-22 have been shown to display proinflammatory activity (213). Th17 cells may mediate protection against TB via IL-17-induced neutrophil recruitment, which produce chemokines for recruitment of protective Th1 cells (214). IL-22 producing cells have been demonstrated to be essential for effective host defence against bacterial infections (215). A study by Dhiman *et al.* reported that *M.tb* growth in human macrophages was restricted by IL-22 producing cells via enhanced phagolysosomal fusion (216). We therefore included IL-17 and IL-22 in our panel. **Table 3** shows a summary and hypothesized roles of the immune parameters thought to be important in the immunobiology of TB.

Effector molecules	Function	Target cells
IFN- $\gamma$	-Activation of macrophages for control of intracellular <i>M.tb</i>	-Macrophages -T cells
IL-2	-Maintenance and differentiation of T and NK cells	-Memory T cells
TNF- $\alpha$	-Formation and maintenance of granuloma structures	-Macrophage
IL-17	-Recruitment of neutrophils and (indirectly) Th1 cells to the site of infection	-Neutrophils -T cells -Macrophages
IL-22	- Limits <i>M.tb</i> growth in macrophages by enhanced phagosome and lysozyme fusion	- $\gamma\delta$ T cells -Macrophages
IL-13	-Control of the effector response against <i>M.tb</i> infection	-Macrophages

**Table 3:** Summary of the immune parameters selected as candidate immune correlates of risk of TB diseases for evaluation in our infant cohort studies.

### 3.1.2 Configuration of the flow cytometer

We aimed to measure the 10 selected phenotypic and functional markers with a BD LSRII flow cytometer. Our instrument comprises 3 lasers and 14 detectors: Red (3 detectors), Blue (8 detectors) and Violet (3 detectors). This configuration facilitates the assessment of a PFC panel that incorporates up to 12 parameters (excluding the side scatter (SCC) and forward scatter (FSC)). The detectors and filters for the cytometer are configured to measure light emission of different fluorochromes. Each fluorochrome has a distinct emission spectrum detectable by a predefined detector; this can be exploited for polychromatic analysis.

Fluorochrome brightness index score (BIS), a relative indication of fluorescence intensity above the background (1=dim, 5=brightest) is an important variable to consider when designing PFC panels. For example, highly expressed markers are easily resolved when stained with antibody-fluorochrome conjugates of low BIS whereas, lowly expressed markers need to be conjugated to fluorochromes of high BIS to be clearly resolved. Detailed settings of the cytometer and fluorochrome BIS are provided in **Table 4**. We carefully considered these settings in the selection of optimal antibody-fluorochrome combinations during our optimization experiments, which are described in the results section of this chapter.

Laser	Detector	LP (nm)	BP (nm)	Fluorochrome	Fluorochrome Brightness Index Score
Blue (488nm)	FSC		488/10		
	G-SSC				
	A	735	780/60	PE-Cy7	4
	B	685	695/40	PerCP-Cy5.5	3
	C	635	670/14	PE-Cy5, PerCP	3, 2
	D	600	610/20	PE-Texas Red	NR
	E	550	576/26	PE	5
	F	505	530/30	FITC	3
Violet (405nm)	A	630	655/8	QDot 655	NR
	B	505	605/12	QDot 605, Pac Orange	NR, 1
	C		440/40	Pac Blue	1
Red (633nm)	A	735	780/60	APC-Cy7	2
	B	690	720/40	Alexa Fluor 700	2
	C		660/20	APC, Alexa Fluor 647	5, 5

**Table 4: BD LSR II configuration and fluorochrome brightness index score:** The LSR II has 3 lasers and 14 detectors. Each detector has a filter; long pass (LP) and band pass (BP) that determine the wavelength at which the fluorochrome emission signal is optimally detected. Fluorochromes that may be used for the different detectors and their associated BIS are also indicated (1=dim, 5=brightest). The BIS for PE-Texas Red, QDots-655 and -605 are not reported (NR) (<http://www.ebioscience.com/ebioscience/appls/Dyes.htm>).

## 3.2 Materials and Methods

### 3.2.1 Short-term whole blood stimulation and cryopreservation

To optimize our 8-colour ICS panel, we used an assay system previously developed in our laboratory (178). In the assay, whole blood is stimulated either with BCG, or SEB (positive control) or left unstimulated (negative control). To all assay conditions, co-stimulatory antibodies of anti-CD28 and anti-CD49d (both from BD Biosciences) are added at a final concentration of 1µg/mL (178). The whole blood is incubated at 37°C for 12 hours, and Brefeldin-A (10µg/mL, Sigma) is added for the last 5 hours of incubation to capture cytokines intracellularly. Upon completion of incubation, the whole blood is harvested with EDTA (2µM, Sigma). Subsequently, red blood cells are lysed and white cells fixed with FACS lysing solution (BD Biosciences). The white cells are then pelleted, followed by cryopreservation in 10% DMSO (Sigma) in fetal calf serum (BioWest).

### **3.2.2 Antigens**

Bacillus Calmette-Guérin (BCG, Danish 1331, Statens Serum Institut, Copenhagen) was prepared from the vaccine vial by reconstituting with RPMI, and is used at a final concentration of  $1.2 \times 10^6$  CFU/mL. This dose was shown to be optimal in earlier experiments (178). Streptococcal enterotoxin B (SEB, Sigma), a positive control, is used at a final concentration of 10 µg/mL.

### **3.2.3 Study participants and blood collection**

Healthy adults were recruited at the University of Cape Town, South Africa. Healthy 10-week old infants who had received BCG vaccine at birth were recruited at SATVI's Worcester clinical site. The protocol was approved by the University of Cape Town Research Ethics Committee, and study conduct was guided by good clinical practice guidelines; this included written informed consent from the participants. Mothers gave consent on behalf of their infants. From each adult donor, 16 mL blood was collected in heparinized tubes. From each healthy infant, 8 mL blood was collected in heparinized tubes. After collection, blood was immediately transferred into pre-prepared tubes containing co-stimulatory antibodies with or without antigens. The blood stimulation and cryopreservation processes are described above.

### **3.2.4 Intracellular cytokine staining assay and flow analysis**

The analysis of fixed white blood cells involves ICS and analysis by PFC.

The following optimisation steps were addressed:

- Optimal antibody-fluorochrome combinations
- Optimal PMT voltage
- The flow cytometer's lower limit of detection
- Fluorescence minus one (FMO)
- Intra-assay coefficient of variation
- Optimal gating strategy
- Pilot studies with samples from healthy adult volunteers
- Pilot studies with samples from healthy 10-week old infants



- T cell event counts in samples from healthy 10-week old infants
- Minimum T cell numbers for reliable analysis of samples from healthy 10-week old infants

### 3.3 Results

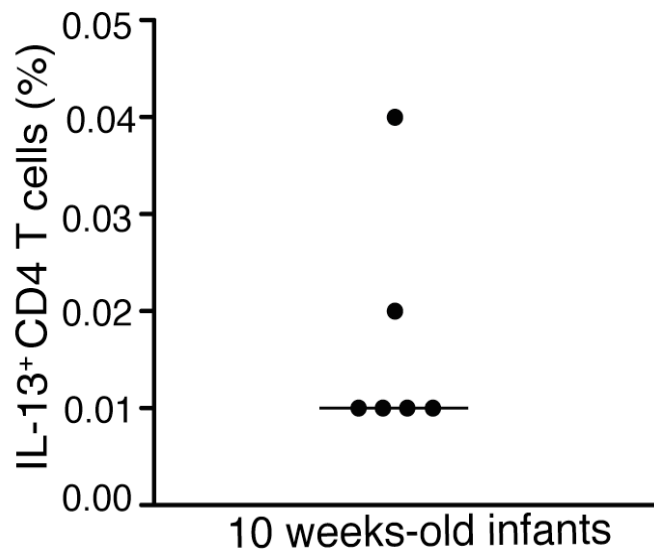
#### 3.3.1 Optimal antibody-fluorochrome combinations

Previously, our laboratory has tested combinations of the following fluorochrome-antibodies: CD3-Pac Blue, CD4-Cy5.5PerCP, CD8-FITC, CD8-APC, CD8-Pac-Orange, CD8-Cy5.5PerCP, CCR7-APC, CD45RA-Cy7PE, IL-13 FITC, IL-2-FITC, IL-2-Alexa610 PE, IFN- $\gamma$  Alexa 700, TNF- $\alpha$ -Cy7PE, IL-17-PE, IL-17-Alexa 647, IL-22-PE, IL-22-APC and  $\gamma\delta$ TCR-APC (130, 178, 212). Additional experiments for selection of the final panel are presented here.

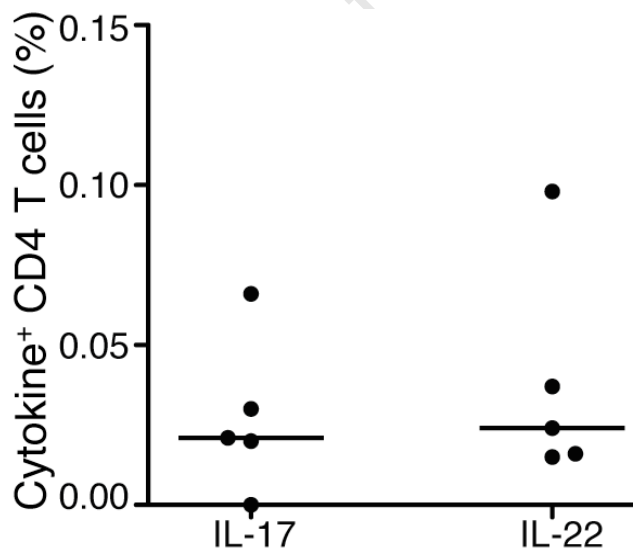
##### 3.3.1.1 BCG-induced expression of IL-13, IL-17 and IL-22

For inclusion in the panel, we wished to ensure that expression of the cytokine was of sufficient magnitude for detection within our whole blood assay system. Our first aim was to measure the frequencies of BCG-induced IL-13, IL-17 and IL-22 expressing T cells in whole blood from BCG-vaccinated infants, incubated with BCG for 12 hours. Unlike IFN- $\gamma$ , TNF- $\alpha$  and IL-2, our laboratory had not previously measured IL-13, IL-17 and IL-22 in infants using this assay system. We first measured BCG-induced IL-13 frequencies using a 4-colour panel: CD3-PacBlue, CD4-Cy5.5PerCP, CD8-APC and IL-13-FITC. We then measured the frequencies of BCG-induced IL-17 and IL-22 using a 5-colour panel: CD3-PacBlue, CD4-Cy5.5PerCP, CD8-FITC, IL-17-Alexa 647 and IL-22-PE. The median frequency for BCG-induced IL-13<sup>+</sup>CD4<sup>+</sup> was 0.01% (n=6, **Figure 11**), whereas the median frequencies for BCG-induced IL-17<sup>+</sup> CD4<sup>+</sup> and IL-22<sup>+</sup>CD4<sup>+</sup> were 0.021% and 0.024%, respectively (n=5, **Figure 12**). We concluded that BCG-induced IL-13<sup>+</sup> CD4<sup>+</sup> T cell frequencies were too low, both in infant and adult (data not shown) samples, to yield statistically reliable data for analysis, given anticipated cell numbers available for analysis. BCG-induced IL-17<sup>+</sup> CD4<sup>+</sup> and IL-22<sup>+</sup> CD4<sup>+</sup> T cells were measureable, although at lower frequencies

than previously shown in adults (212), and were included for further assessment.



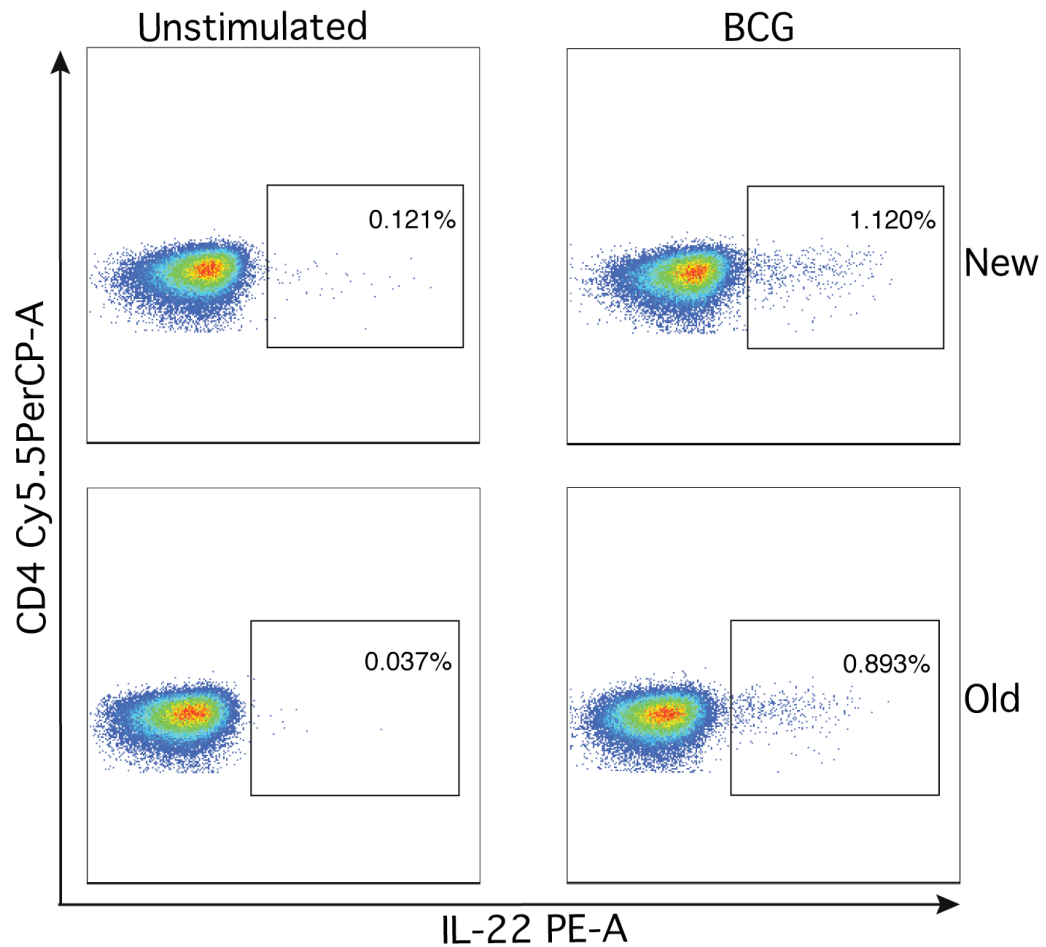
**Figure 11: Frequency of BCG-induced IL-13<sup>+</sup> CD4<sup>+</sup> T cells in infants.** Whole blood from six 10-week old infants was stimulated with BCG for 12 hours, red blood cells lysed, white cells fixed, followed by cryopreservation. An ICS assay was performed to determine the BCG-induced frequencies of IL-13 in CD4<sup>+</sup> T cells. Backgrounds were subtracted from the respective BCG-specific responses.



**Figure 12: Frequencies of BCG-induced IL-17<sup>+</sup> and IL-22<sup>+</sup> CD4<sup>+</sup> T cells in infants.** Whole blood from five 10-week old infants was stimulated with BCG for 12 hours, red blood cells lysed, white cells fixed, followed by cryopreservation. An ICS assay was performed to determine the BCG-induced frequencies of IL-17 and IL-22 in CD4<sup>+</sup> T cells. Backgrounds were subtracted from the respective BCG-specific responses.

### 3.3.1.2 IL-22-PE exclusion

Antibody batch variation may greatly affect staining profiles even though the supplier and clone remain unchanged. After using an old IL-22-PE antibody for the BCG-induced IL-22 experiments described above (**Figure 12**) (this antibody had been previously titrated), we ordered a fresh vial of IL-22-PE antibody. IL-22-PE antibodies from the fresh and old vials (both clone 142928) from R&D systems were utilized for staining and the outcome compared directly. Adult BCG-stimulated whole blood cells were stained with CD3-PacBlue, CD4-Cy5.5PerCP, CD8-FITC and IL-22-PE (old and new). Although both antibodies were stored under similar conditions, staining with the fresh IL-22-PE antibody vial revealed a high background frequency of 0.121%, compared to a background frequency of 0.037% after staining with the old IL-22-PE antibody vial (**Figure 13**). We concluded that the use of different IL-22-PE antibody vials may compromise analysis of IL-22, and therefore excluded this marker from further analysis. Although both antibodies were stored under similar conditions, staining with the fresh IL-22-PE antibody vial revealed a high background frequency of 0.121%, compared to a background frequency of 0.037% after staining with the old IL-22-PE antibody vial (**Figure 13**). We concluded that the use of different IL-22-PE antibody vials may compromise analysis of IL-22, and therefore excluded this marker from further analysis. Although more experiments would have been appropriate to confirm the variability in staining with the IL-22-PE antibody, we chose not to because the role of IL-22 cytokine in TB immunity remains poorly defined. As we had another flow cytometric channel available for analysis, we chose to assess  $\gamma\delta$  T cells because a role for these cells in host responses to TB is relatively well established (200). The anti- $\gamma\delta$  antibody has been tested and shown to work well in our laboratory (212).

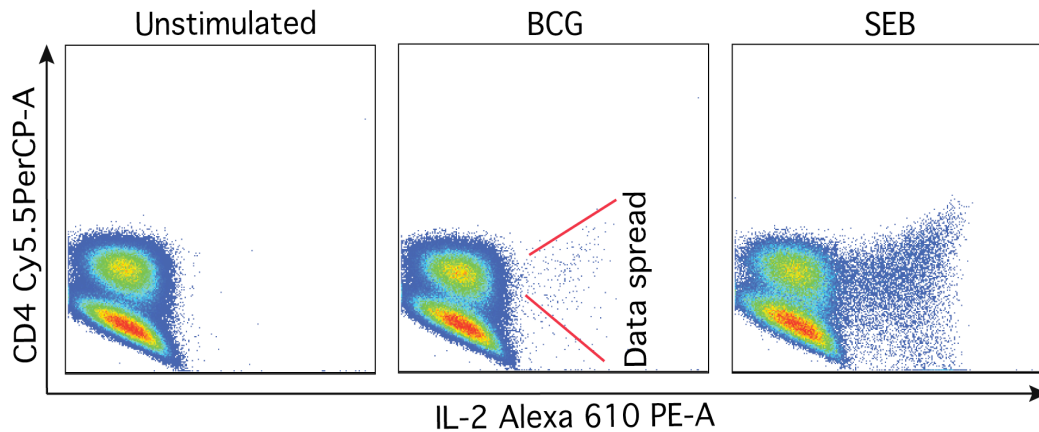


**Figure 13: IL-22-PE antibody staining.** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours, and Brefeldin-A added in the last 5 hours to capture intracellular cytokines. The ICS assay was performed to directly compare new and old IL-22-PE Abs. For flow analysis, singlets cells were first selected, then lymphocytes, and finally CD3<sup>+</sup> T cells were differentiated into CD4<sup>+</sup> and CD8<sup>+</sup> T cells. We compared the staining observed with the fresh and old IL-22-PE antibody vials in CD4 T cells.

### 3.3.1.3 IL-2-Alexa 610PE exclusion

We evaluated the compatibility of the IL-2-Alexa 610 PE antibody in our proposed PFC panel. Our laboratory had previously observed spectral overlap between Cy5.5PerCP and Alexa 610 PE. The overlap may however vary with the antibody-fluorochrome combinations included in the panel. We used unstimulated and stimulated fixed whole blood derived from an adult volunteer, and stained the samples with CD3-PacBlue, CD4-Cy5.5PerCP, CD8-FITC, and IL-2-Alexa 610 PE. We noted a high compensation between Cy5.5PerCP and Alexa 610 PE, which resulted in large data spread (**Figure 14**). Large data spread may lead to detection of artifacts in other channels of

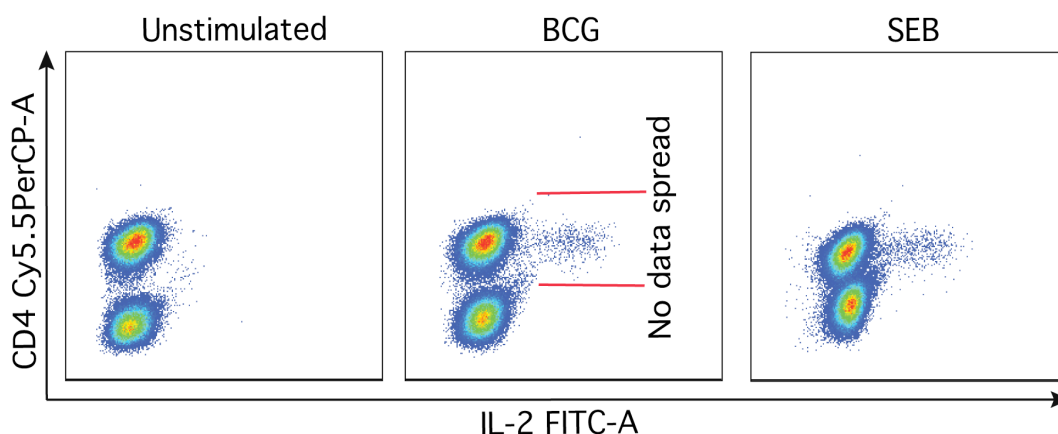
interest. We therefore excluded this antibody-fluorochrome combination from our proposed panel.



**Figure 14: IL-2-Alexa 610 PE antibody staining.** Whole blood sample derived from an adult volunteer was left unstimulated, stimulated with BCG or SEB for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The ICS assay was performed with CD3-Pac Blue, CD4-Cy5.5PerCP, CD8-FITC and IL-2-Alexa 610 PE. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

#### 3.3.1.4 IL-2-FITC inclusion

Next, we aimed to evaluate whether the IL-2-FITC antibody would be compatible with our proposed PFC panel. We used unstimulated and stimulated fixed whole blood cells derived from an adult volunteer and stained the cells with CD3-PacBlue, CD4-Cy5.5PerCP, CD8-Qdot 605, and IL-2-FITC. We did not observe any data spread when IL-2-FITC was evaluated against CD4-Cy5.5PerCP (**Figure 15**). We therefore included this antibody in our proposed PFC panel.

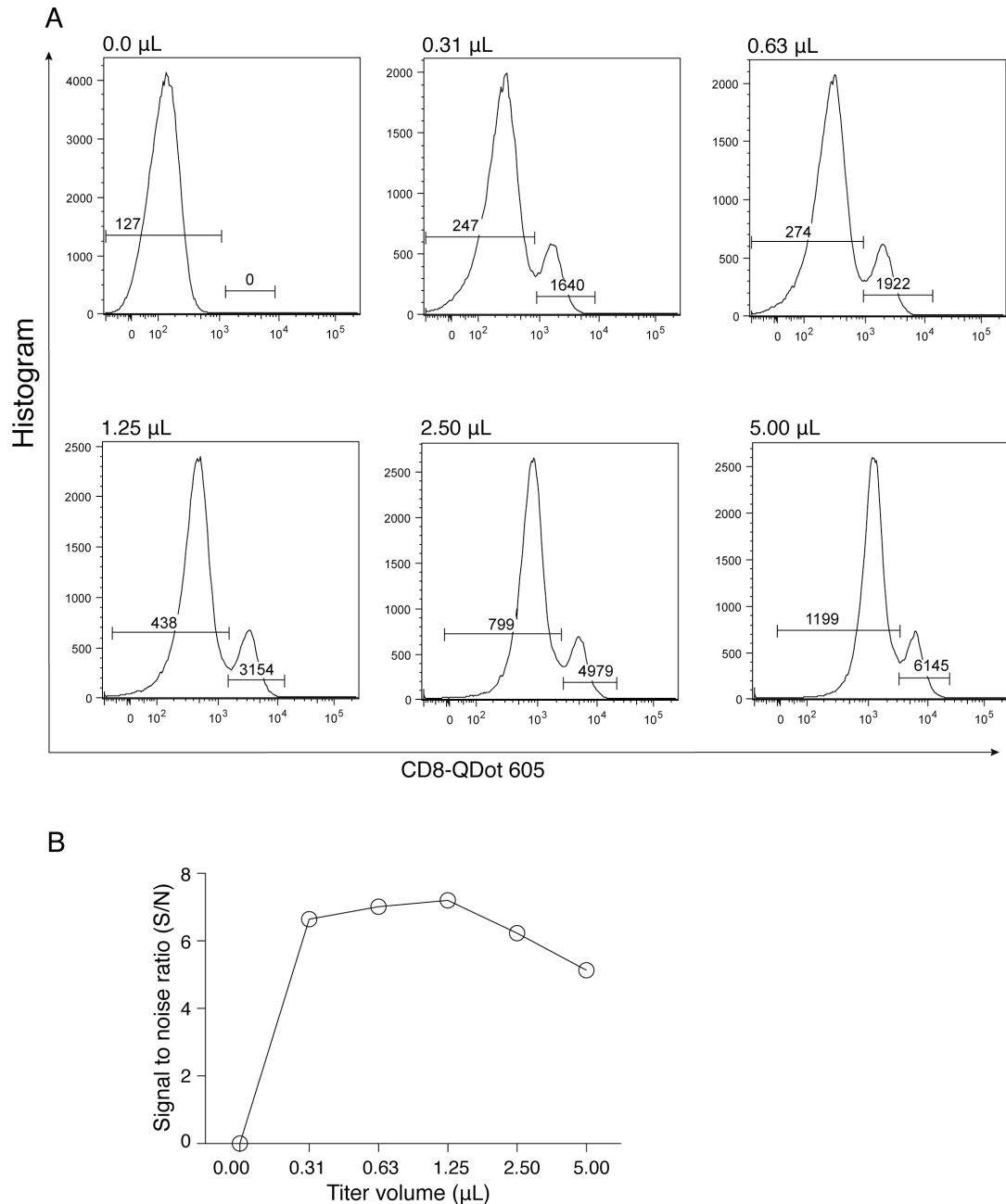


**Figure 15: IL-2-FITC antibody staining.** Whole blood sample derived from an adult volunteer was left unstimulated, stimulated with BCG or SEB for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The ICS assay was performed with CD3-Pac Blue, CD4-Cy5.5PerCP, CD8-Qdot 605 and IL-2-FITC. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells..

### 3.3.1.5 CD8-QDot 605 inclusion

Next, we decided to directly compare the outcome after staining with two candidate antibodies: CD8-Pac Orange and CD8-QDot 605. In our previous experiments, we had successfully used CD8-FITC and CD8-APC antibodies. However, we wished to use the bright FITC and APC fluorochromes for IL-2 and  $\gamma\delta$ TCR, respectively. The IL-2-FITC and  $\gamma\delta$ TCR-APC antibodies had been previously tested in a different panel in our laboratory (212). To compare CD8-Pac Orange and CD8-QDot 605 antibodies, we first titrated a newly introduced CD8-QDot 605 antibody. It is important to titrate new antibody-fluorochrome combinations to ensure that the chosen antibody concentrations yield an optimal signal compared with background noise, and have reached a saturation point (195). Antibody concentrations that are too low result in suboptimal signal. Antibody concentrations that are too high are wasteful and expensive, and may result in non-specific binding of the antibody and consequently false positive results. We used BCG-stimulated whole blood from an adult participant for the titration experiment. Six different CD8-QDot 605 antibody concentrations were evaluated. The signal to noise (S/N) ratio, a measure of the signal intensity was computed for all the titration volumes used (**Figure 16**). We concluded that the 0.63 $\mu$ L (0.0063 $\mu$ M/mL) antibody titer volume was optimal for staining in our panel as it yields the highest S/N

ratio with the lowest titer volume. Below the optimal chosen titer volume, the S/N ratio starts getting lower. Similar optimal titer volumes were noted for the CD4-QDot 605 antibody.

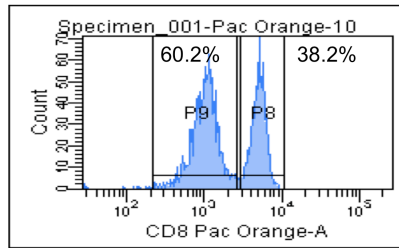


**Figure 16: CD8-QDot 605 antibody titration plots.** Six different CD8-QDot 605 antibody titer volumes were evaluated using BCG-stimulated whole blood derived from an adult volunteer and stained with CD3-Pac Blue, CD4-Cy5.5PerCP and CD8-QDot 605. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The MFI of CD8<sup>+</sup> (signal) and CD8<sup>-</sup> (noise) T cells was assessed at different antibody concentrations (**A**). The optimal titer volumes were assessed by signal to noise ratio (S/N) titration curves (**B**).

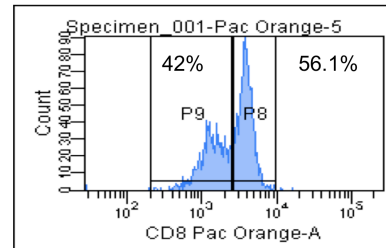
After establishing the optimal titration volume for CD8-QDot 605, we titrated CD8-Pac Orange (**Figure 17**), and then proceeded to compare resolution of the CD8 T cell subset after staining with either CD8-Pac Orange or CD8-QDot 605 antibodies in our proposed PFC panel. Staining for markers conjugated with brighter fluorochromes such as PE, APC, Cy7PE and QDots, result in better separation of negative and positive cell populations. We stained adult BCG-stimulated whole blood cells with CD3-Pac Blue, CD4-Cy5.5PerCP and CD8-QDot 605 or CD8-Pac Orange. The CD8-QDot 605 antibody showed a brighter staining that easily resolved the CD8<sup>+</sup> and CD8<sup>-</sup> cell populations. The CD8-Pac Orange antibody showed a dim staining with poor separation of the CD8<sup>+</sup> and CD8<sup>-</sup> T cell populations (**Figure 18**). We therefore excluded CD8-Pac Orange, and rather selected CD8-QDot 605 antibody for our proposed panel.



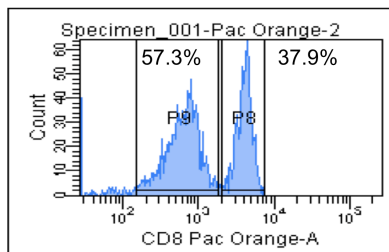
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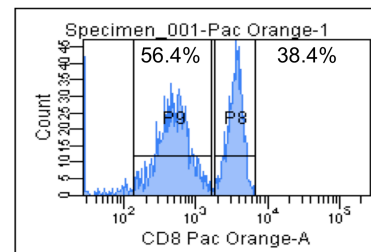
At titre volume of 10 $\mu$ l  
Signal: noise ratio=p8/p9  
=5100.7/1067.5  
=4.78



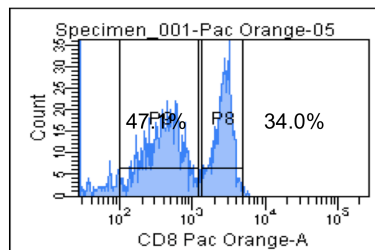
At titre volume of 5 $\mu$ l  
Signal: noise ratio=p8/p9  
=3936.2/1402.8  
=2.81



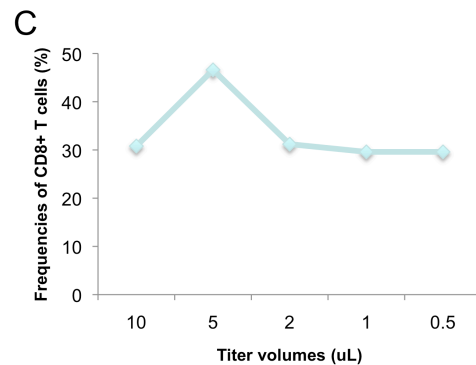
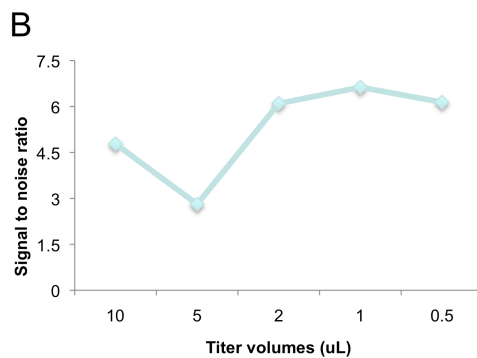
At titre volume of 2 $\mu$ l  
Signal: noise ratio=p8/p9  
=4069.9/667.6  
=6.10



At titre volume of 1 $\mu$ l  
Signal: noise ratio=p8/p9  
=3573.3/539.1  
=6.63

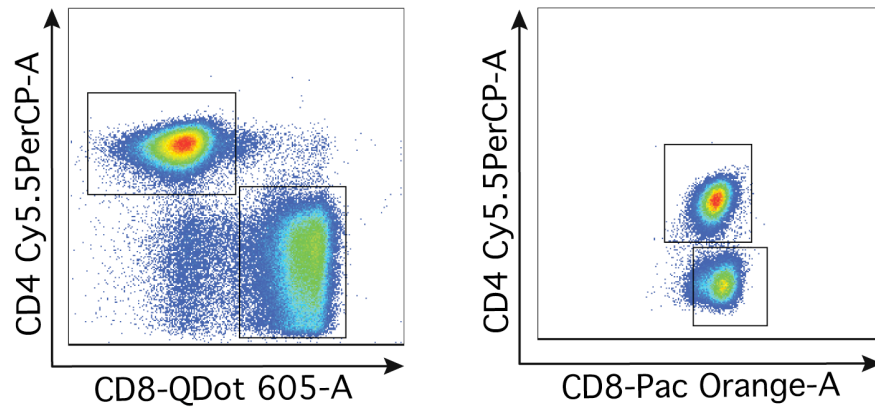


At titre volume of 0.5 $\mu$ l  
Signal: noise ratio=p8/p9  
=2706.8/440.6  
=6.14



**Figure 17: CD8-Pac Orange antibody titration plots.** Five different CD8-Pac Orange antibody titer volumes were evaluated using BCG-stimulated whole blood derived from an adult volunteer and stained with CD3-Pac Blue, CD4-Cy5.5PerCP and CD8-Pac Orange (A).

For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Curves for the signal to noise ratio (MFI of CD8<sup>+</sup> as the signal and CD8<sup>-</sup> as the noise ) (B) and frequencies of CD8<sup>+</sup> T cells (C), against titration volumes were generated. The 2 $\mu$ L and 5 $\mu$ L volumes appeared optimal and were further tested in a full 8-colour panel. The 5 $\mu$ L volume gave a better performance (data not shown) and was subsequently used.



**Figure 18: CD8-QDot 605 and CD8-Pac Orange staining.** Whole blood sample derived from an adult volunteer was stimulated with BCG for 12 hours and stained with CD3-PacBlue, CD4-Cy5.5PerCP, CD8-Qdot 605 or CD8-Pac Orange. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The CD8-Qdot 605 antibody stain was bright and resulted in clearly resolved CD8<sup>+</sup> and CD8<sup>-</sup> T cells (left plot). The CD8-Pac Orange antibody stain was dim and resulted in poorly resolved CD8<sup>+</sup> and CD8<sup>-</sup> T cells (right plot).

### 3.3.1.6 Final antibody-fluorochrome combination

Our next aim was to select the final antibody-fluorochrome combinations. We chose CD3-PacBlue, CD4-Cy5.5PerCP CD8-QDot 605,  $\gamma\delta$ T-APC, TNF-Cy7PE, IFN- $\gamma$ -Alexa 700, IL-2-FITC and IL-17-PE antibody-fluorochrome combinations (Table 5). This combination provided the optimal 8-colour PFC panel.

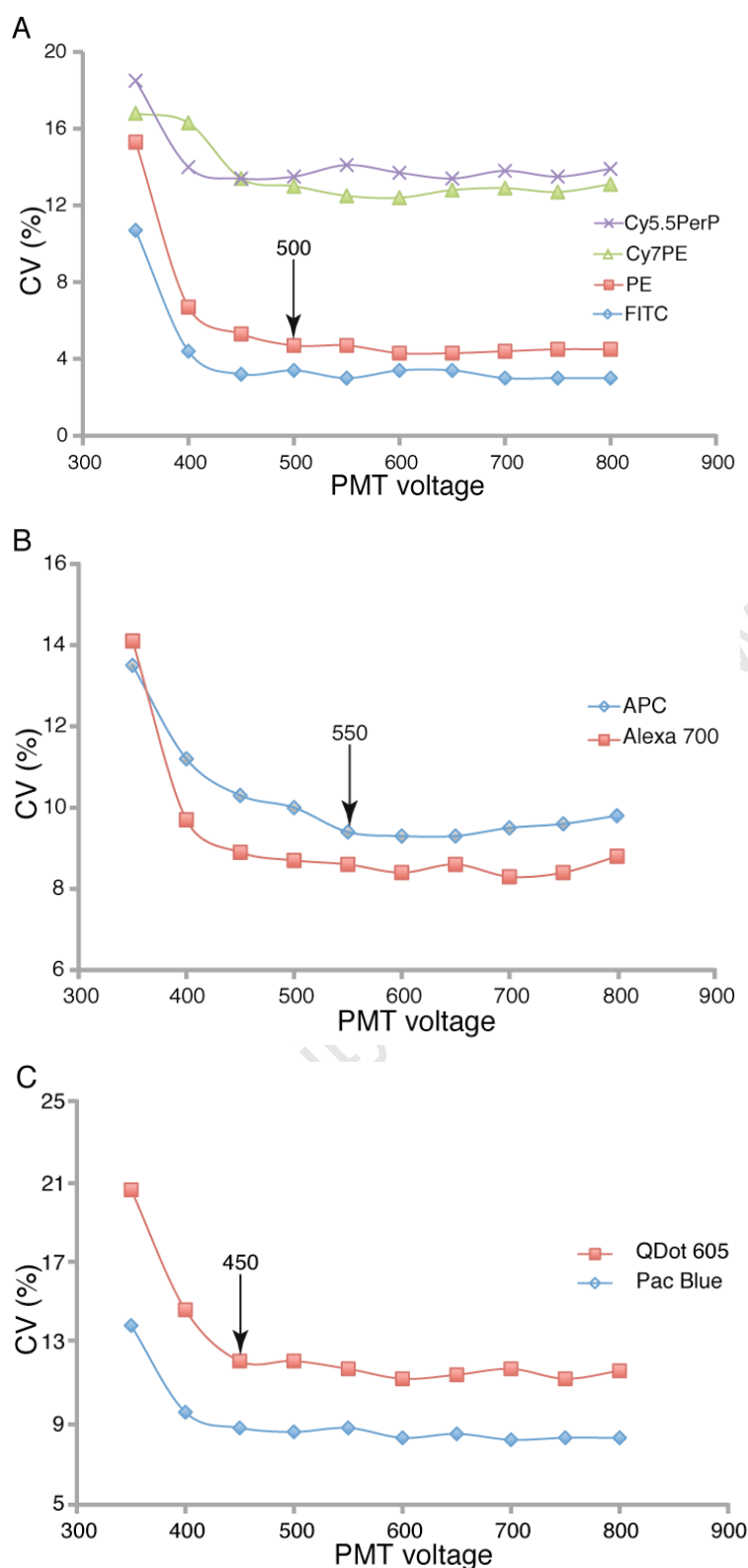
Analyte	Detector	Fluorochrome	Manufacturer	Cat.No
CD3	Violet, C	Pac Blue	BD	558117
CD4	Blue, C	Cy5.5PerCP	BD	332772
CD8	Violet, B	QDot 605	Invitrogen	Q10009
$\gamma\delta$ TCR	Red, C	APC	BD	555718
TNF- $\alpha$	Blue, A	Cy7PE	BD	557647
IL-2	Blue, F	FITC	BD	340448
IFN- $\gamma$	Red, B	Alexa Fluor 700	BD	544699
IL-17	Blue, E	PE	EBioscience	12-7178-71

**Table 5:** Detailed description of the antibody-fluorochrome combinations for the final panel and the detectors on the LSR II cytometer for assessing the markers. Manufacturer's names and catalogue numbers are provided.

### 3.3.2 Optimal photomultiplier tube (PMT) voltage

#### 3.3.2.1 Evaluating the baseline PMT settings

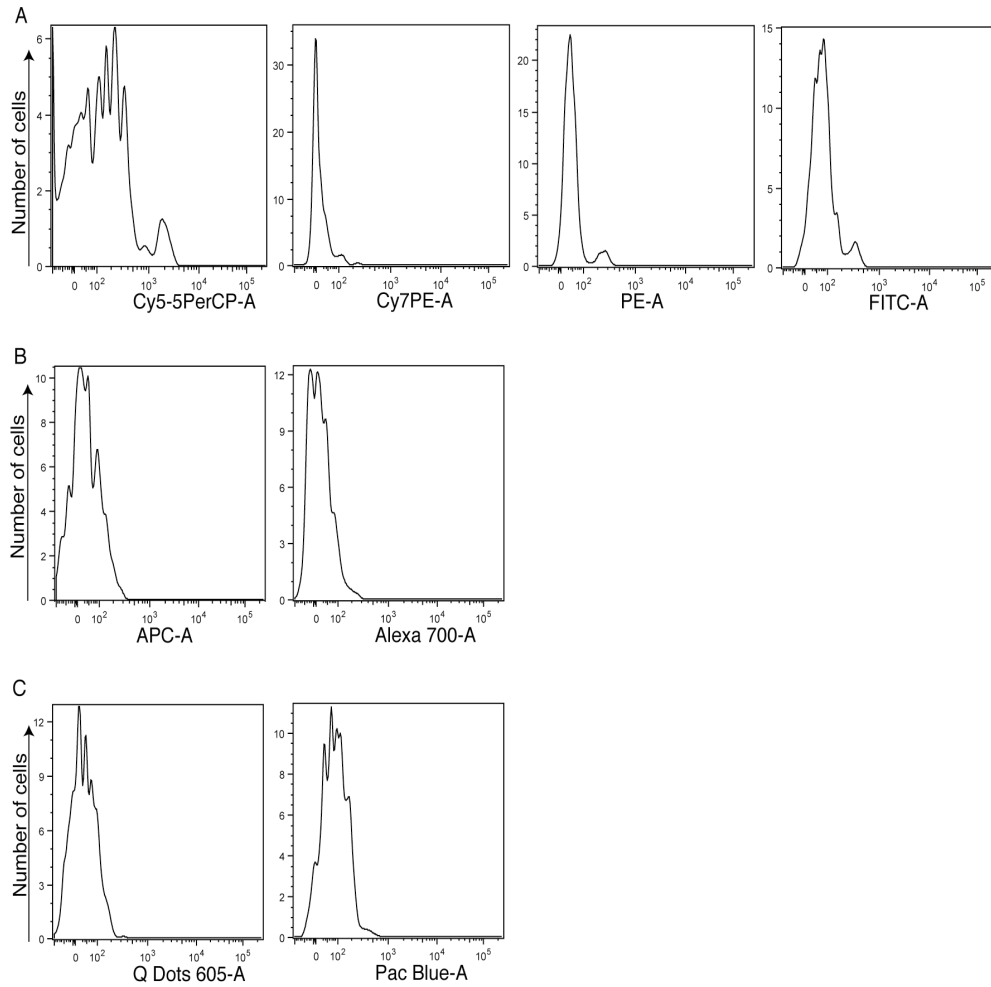
Next, we aimed to establish the optimal PMT voltages for the channels in our final panel. Optimal PMT voltages ensure the fluorescence signal that is detected allows for maximum resolution for antibody staining (217). Lower than optimal PMT voltage results in stained markers of interest falling below the scale, while higher voltage results in the markers being off the scale. To establish the optimal PMT voltages, we first used 6 peak 3.0-3.4mM spherotech rainbow calibration particles (BD Biosciences). These particles fluoresce in all channels of LSR II cytometer (218). For each channel in our final PFC panel, we measured the coefficient of variation (CV, as a %) of the fluorescence of these particles at different PMT voltages (350-800). We then graphed the CV against the PMT voltages. On the graph, the baseline voltage was determined by selecting the lowest voltage where the CV showed stability. The baseline voltages for blue laser channels were: 450 for Cy5.5PerCP, 500 for both Cy7PE and PE, and 450 for FITC (**Figure 19A**). Baseline voltages for the red laser channels APC and Alexa Fluor 700 were 500 and 450, respectively (**Figure 19B**). The baseline voltages for the violet laser channels were 450 and 400 for QDot 605 and Pac Blue, respectively (**Figure 19C**). These voltages were later evaluated for optimal resolution by use of unstained cells and single stained mouse kappa compensation beads. We successfully established baseline voltages that showed minimal CV in fluorescence of the calibration beads.



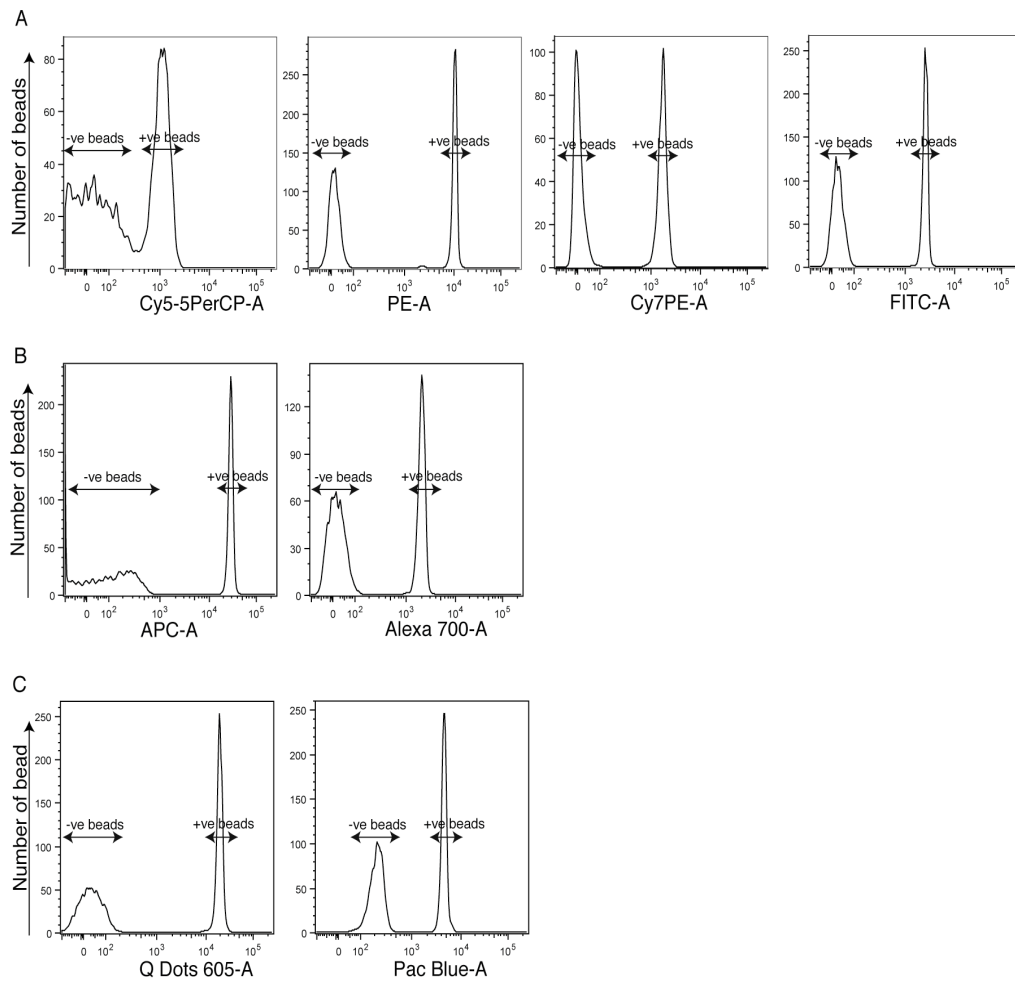
**Figure 19: Baseline PMT voltages for the channels assessed.** In a 5mL falcon tube, 1mL of sheath fluid was added followed by a single drop of 6 peaks 3.0-3.4mM spherotech rainbow calibration particles. The mixture was vortexed and acquired on an LSR II cytometer at different PMT voltage settings (350 to 850). For each channel, we selected a single peak on the extreme left of the fluorescence plot when the PMT voltage was lowest. The fluorescence CV (%) for the peak was recorded at different PMT voltage settings for the blue laser (A), red laser (B) and violet laser (A). The arrows on the plots are examples to show the baseline PMT voltages selected for PE (A), APC (B) and QDot 605 (C).

### 3.3.2.2 Establishing the final PMT settings

The second step in PMT voltage optimization was to evaluate and adjust the selected baseline voltages to give an on scale negative (unstained) and positive (antibody stained) signal. Generally, a negative population should lie within the first decade of the x-axis scale. To adjust the baseline voltages, we used BCG-stimulated and fixed whole blood cells from a single adult participant. We then used the adjusted voltages to assess the negative and positive resolution of anti-mouse kappa-comp beads (BD Biosciences), labelled with the specific fluorochrome-conjugated antibodies for the 8-colour panel. Using unstained cells, we selected for lymphocytes and adjusted the voltages to give an on scale signal for the blue laser (**Figure 20A**), red laser (**Figure 20B**) and violet laser (**Figure 20C**). The adjusted voltages were then evaluated on single stained anti-mouse kappa compensation beads: blue laser (**Figure 21A**), red laser (**Figure 21B**) and violet laser (**Figure 21C**). We established that the optimal voltages were: 650 for both Cy5-5PerCP and Cy7PE-A, 500 for PE, 620 for FITC (blue laser), 600 for both APC and Alexa Fluor 700 (red laser), 550 for QDot 605 and 450 for Pac Blue (violet laser). We concluded that the selected voltages were optimal to provide maximum resolution for the positive and negative markers of interest in our final PFC panel.



**Figure 20: Final PMT voltage settings I.** Whole blood from an adult volunteer was stimulated with BCG for 12 hours. For flow analysis, no antibody staining was performed. Lymphocytes were selected by forward scatter-area (FSC-A) against side scatter-area (SSC-A). For each channel of our 8-colour panel, PMT voltages were adjusted to the established baseline settings. The PMT voltages were then adjusted to allow for the unstained lymphocytes to fall within the first and second decade of the scale for the blue laser (**A**), red laser (**B**) and violet laser (**C**).



**Figure 21: Final PMT voltage settings II.** In eight separate 5mL falcon tubes, single drops of negative and positive anti-mouse kappa compensation beads were added. To each of the tubes, the established antibody staining volumes for CD3-PacBlue, CD4-Cy5.5PerCP CD8-QDot 605,  $\gamma\delta$ T-APC, TNF-Cy7PE, IFN- $\gamma$ -Alexa 700, IL-2-FITC and IL-17-PE were added separately. For flow analysis, the bead population were selected by forward scatter-area (FSC-A) against side scatter-area (SSC-A). For each antibody staining, PMT voltages were adjusted to the final PMT voltage settings established using the unstained cells. The final PMT voltage settings showed distinct negative and positive beads population for the blue laser (A), red laser (B) and violet laser (C).

### 3.3.3 The lower limit of detection for the LSR II flow cytometer

The next step was to establish the lower limit of detection for BCG-induced IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and IL-17, when measured with our LSR II cytometer. The cytometer's lower limit of detection is defined as a limit below which the reliability of measuring the true value is compromised. We used fixed cells from BCG-stimulated whole blood sample from an adult. The stimulated cells were serially diluted with autologous unstimulated cells up to 4096-fold. We then stained the cells with our final 8-color panel, acquired and analyzed for



BCG-induced CD4 T cell cytokine expression. The background (unstimulated) frequencies were subtracted from the BCG-induced frequencies (represented as observed frequencies in **Tables 6-9** below). Expected frequencies were obtained by dividing the undiluted BCG-induced frequencies with the dilution fold. The lower limit of detection for BCG-induced cytokines was evaluated by calculating the magnitude of deviation for the observed from expected values using the following formula:

$$\frac{(\text{Expected} \times \text{dilution factor}) - (\text{Observed} \times \text{dilution factor})}{(\text{Expected} \times \text{dilution factor})} \times 100\%$$

Deviations  $\geq 30\%$  were considered large enough to compromise the reliability of the cytometer. We found that the cytometer's lower limit of detection for IFN- $\gamma$  and IL-2 was about 0.04% (**Tables 6 and 8**). The lower limit of detection for TNF- $\alpha$  and IL-17 was approximately 0.01% (**Tables 7 and 9**), respectively, suggesting that these cytokines could be reliably measured to vary below 0.01%. We also report that the sensitivity of the cytometer decreased as the dilution factor increased (**Figures 22A to D**). We used a short-term whole blood assay with infants' samples to show that median frequencies of BCG-induced T cell IFN- $\gamma$ , IL-2 and TNF- $\alpha$  are approximately 0.1%. In earlier BCG-induced cytokines expression experiments, we established the frequencies of BCG-induced T cell IL-17 were approximately 0.02%. We therefore concluded that the frequencies of BGC-induced T cells cytokines selected in the 8-colour PFC panel are over the limit of detection and hence reliable to measure with our flow cytometer.

CD4 <sup>+</sup> IFN- $\gamma$ <sup>+</sup> (%)				Deviation of observed from expected values (%)	Dilution fold
Observed	Expected	Observed x dilution fold	Expected x dilution fold		
1.428	1.428	1.428	1.428	0.00	1
0.618	0.714	1.236	1.428	13.45	2
0.328	0.357	1.312	1.428	8.12	4
0.168	0.179	1.344	1.428	5.88	8
0.073	0.089	1.168	1.428	18.21	16
0.041	0.045	1.312	1.428	8.12	32
0.030	0.022	1.920	1.428	34.45	64
0.016	0.011	2.048	1.428	43.41	128
0.010	0.006	2.560	1.428	79.27	256
0.005	0.003	2.560	1.428	79.27	512
0.009	0.001	9.216	1.428	>100	1024
0.004	0.001	8.192	1.428	>100	2048
0.004	0.000	16.384	1.428	>100	4096

**Table 6: Assessment of the LSR II limit of detection for IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup>.** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The BCG sample was serially diluted with the unstimulated sample up to 4096-fold. The ICS assay was performed with the full 8-colour panel. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of BCG-induced IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> was analyzed and corrected for background levels to give the observed frequencies. The observed frequencies in the undiluted sample was presumed to be the same as expected frequencies in the same sample. Subsequently, expected frequencies in the diluted samples were calculated by dividing the expected frequencies in the undiluted sample with the dilution fold. Then, the observed and expected frequencies were multiplied by the dilution fold and the percentage deviation between the two frequencies calculated. Deviations  $\geq$  30% were indicative of loss of sensitivity to detect the expected values. The shaded expected value (0.041%) show the limit of detection for this channel.

TNF- $\alpha$ <sup>+</sup> CD4 <sup>+</sup> (%)				Deviation of observed from expected values (%)	Dilution fold
Observed	Expected	Observed x dilution fold	Expected x dilution fold		
0.659	0.659	0.659	0.659	0.000	1
0.249	0.329	0.498	0.659	24.43	2
0.149	0.165	0.596	0.659	9.56	4
0.083	0.082	0.664	0.659	0.76	8
0.031	0.041	0.496	0.659	24.73	16
0.020	0.021	0.640	0.659	2.88	32
0.010	0.010	0.640	0.659	2.88	64
0.009	0.005	1.152	0.659	74.81	128
0.005	0.003	1.280	0.659	94.23	256
0.003	0.001	1.536	0.659	>100	512
0.003	0.001	3.072	0.659	>100	1024
0.002	0.000	4.096	0.659	>100	2048
0.003	0.000	12.288	0.659	>100	4096

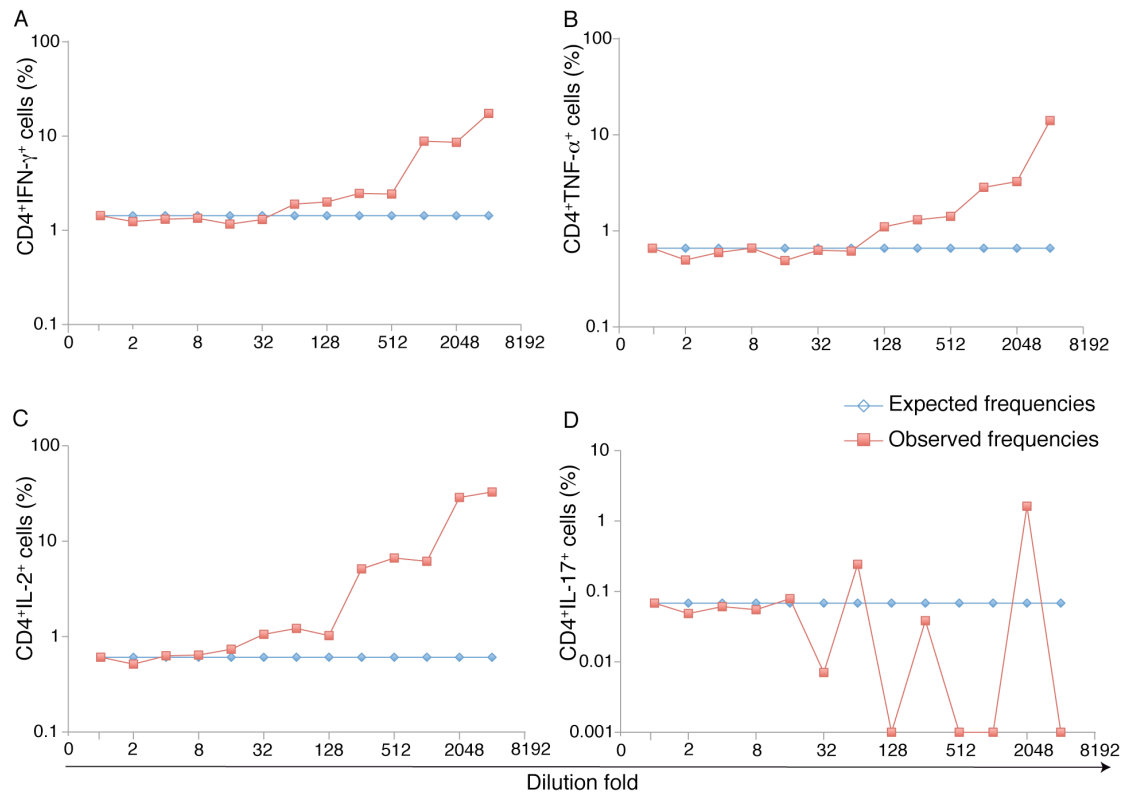
**Table 7: Assessment of the LSR II limit of detection for TNF- $\alpha$ <sup>+</sup>CD4<sup>+</sup>.** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The BCG sample was serially diluted with the unstimulated sample up to 4096 fold. The ICS assay was performed with the full 8-colour panel. For flow analysis, singlets cells were first selected and then lymphocytes. Thereafter, we selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of BCG-induced TNF- $\alpha$ <sup>+</sup>CD4<sup>+</sup> was analyzed and corrected for background levels to give the observed frequencies. The observed frequencies in the undiluted sample was presumed to be the same as expected frequencies in the same sample. Subsequently, expected frequencies in the diluted samples were calculated by dividing the expected frequencies in the undiluted sample with the dilution fold. Then, the observed and expected frequencies were multiplied by the dilution fold and the percentage deviation between the two frequencies calculated. Deviations  $\geq$  30% were indicative of loss of sensitivity to detect the expected values. The shaded expected value (0.01%) show the limit of detection for this channel.

IL-2 <sup>+</sup> CD4 <sup>+</sup> (%)				Deviation of observed from expected values (%)	Dilution fold
Observed	Expected	Observed x dilution fold	Expected x dilution fold		
0.607	0.607	0.607	0.607	0.00	1
0.257	0.304	0.514	0.607	15.32	2
0.157	0.152	0.628	0.607	3.46	4
0.080	0.076	0.640	0.607	5.44	8
0.046	0.038	0.736	0.607	21.25	16
0.033	0.019	1.056	0.607	73.97	32
0.019	0.009	1.216	0.607	100.33	64
0.008	0.005	1.024	0.607	68.70	128
0.020	0.002	5.120	0.607	>100	256
0.013	0.001	6.656	0.607	>100	512
0.006	0.001	6.144	0.607	>100	1024
0.014	0.000	28.672	0.607	>100	2048
0.008	0.000	32.768	0.607	>100	4096

**Table 8: Assessment of the LSR II limit of detection for IL-2<sup>+</sup>CD4<sup>+</sup>.** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The BCG sample was serially diluted with the unstimulated sample up to 4096 fold. The ICS assay was performed with the full 8-colour panel. For flow analysis, lymphocytes were selected followed by singlets cells. We thereafter selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of BCG-induced IL-2<sup>+</sup>CD4<sup>+</sup> was analyzed and corrected for background levels to give the observed frequencies. The observed frequencies in the undiluted sample was presumed to be the same as expected frequencies in the same sample. Subsequently, expected frequencies in the diluted samples were calculated by dividing the expected frequencies in the undiluted sample with the dilution fold. Then, the observed and expected frequencies were multiplied by the dilution fold and the percentage deviation between the two frequencies calculated. Deviations  $\geq 30\%$  were indicative of loss of sensitivity to detect the expected values. The shaded expected value (0.046%) show the limit of detection for this channel.

IL-17 <sup>+</sup> CD4 <sup>+</sup> (%)				Deviation of observed from expected values (%)	Dilution fold
Observed	Expected	Observed x dilution fold	Expected x dilution fold		
0.068	0.068	0.068	0.068	0.00	1
0.024	0.034	0.048	0.068	29.41	2
0.015	0.017	0.060	0.068	11.76	4
0.007	0.009	0.056	0.068	17.65	8
0.005	0.004	0.080	0.068	17.65	16
0.0002	0.002	0.006	0.068	91.18	32
0.004	0.001	0.256	0.068	276.47	64
0.000	0.001	0.000	0.068	>100	128
0.000	0.000	0.038	0.068	>100	256
0.000	0.000	0.000	0.068	>100	512
0.000	0.000	0.000	0.068	>100	1024
0.001	0.000	1.618	0.068	98.53	2048
0.000	0.000	0.000	0.068	>100	4096

**Table 9: Assessment of the LSR II limit of detection for IL-17<sup>+</sup>CD4<sup>+</sup>.** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The BCG sample was serially diluted with the unstimulated sample up to 4096 fold. The ICS assay was performed with the full 8-colour panel. For flow analysis, lymphocytes were selected followed by singlets cells. We thereafter selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of BCG-induced IL-17<sup>+</sup>CD4<sup>+</sup> was analyzed and corrected for background levels to give the observed frequencies. The observed frequencies in the undiluted sample was presumed to be the same as expected frequencies in the same sample. Subsequently, expected frequencies in the diluted samples were calculated by dividing the expected frequencies in the undiluted sample with the dilution fold. Then, the observed and expected frequencies were multiplied by the dilution fold and the percentage deviation between the two frequencies calculated. Deviations  $\geq 30\%$  were indicative of loss of sensitivity to detect the expected values. The shaded expected value (0.01%) show the limit of detection for this channel.



**Figure 22: Flow cytometer's limit of detecting BCG-induced cytokines** Whole blood from an adult volunteer was left unstimulated or stimulated with BCG for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines. The BCG sample was serially diluted with the unstimulated sample up to 4096 fold. The ICS assay was performed with the full 8-colour panel. For flow analysis, lymphocytes were selected followed by singlets cells. We thereafter selected for CD3<sup>+</sup> T cells from which we selected CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of BCG-induced IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> (A), TNF- $\alpha$ <sup>+</sup>CD4<sup>+</sup> (B), IL-2<sup>+</sup>CD4<sup>+</sup> (C) and IL-17<sup>+</sup>CD4<sup>+</sup> (D) was analyzed and corrected for background levels to give the observed frequencies. The observed frequencies in the undiluted sample was presumed to be the same as expected frequencies in the same sample. Subsequently, expected frequencies in the diluted samples were calculated by dividing the expected frequencies in the undiluted sample with the dilution fold. Then, the observed and expected frequencies were multiplied by the dilutionfold and the frequencies plotted against the dilution fold. The observed and expected frequencies of BCG-induced CD4 T cell cytokines for corresponding dilution fold is detailed in Tables 2-5. The sensitivity of detecting the BCG-induced CD4<sup>+</sup> T cell cytokines decreased with increased dilution.

### 3.3.4 Fluorescence minus one (FMO) analysis

Next, we aimed to assess the spectral overlap for the new 8-colour antibody-fluorochrome combination selected. In PFC, larger panels may result in higher spectral overlap. When spectral overlap is not carefully corrected, there is a greater chance of measuring artifacts. Spectral overlap can be assessed and quantified by use of FMO controls. The FMO controls contain all the antibodies in the panel except the one being assessed (219). Absence of a signal from an FMO control indicates zero spectral overlap from other

channels. A high signal in an FMO control indicates high spectral overlap from other fluorochromes in the panel. To assess for the spectral overlap in our selected panel, fixed BCG-stimulated whole blood cells from an adult participant was split in to 9 aliquots. One of the 9 aliquots was stained with the full 8-color antibody-fluorochrome combination and the signal for each marker was quantified by measuring the frequencies of the stained markers. The other 8 aliquots were each stained with FMO controls and the spectral overlap detected in the control measured. The range for spectral overlap (shaded boxes) detected in our FMO controls was 0 to 0.06% (**Table 10**). We concluded that the spectral overlap detected for all 8 channels was low and would not compromise the reliability of the panel to evaluate for markers of interest.

FMO	Signal of the immunological marker detected (%)							
	CD3	CD4	CD8	$\gamma\delta$ TCR	IL-2	IL-17	IFN- $\gamma$	TNF- $\alpha$
Pac Blue	0.00	42.02	42.02	3.00	0.27	0.14	0.78	0.28
Cy5.5PerCP	85.11	0.30	39.35	3.00	0.23	0.15	0.87	0.26
Q Dots 605	82.67	41.19	0.00	3.00	0.25	0.15	0.91	0.28
APC	83.00	40.17	37.83	0.05	0.24	0.16	0.81	0.26
FITC	85.50	42.60	39.45	3.29	0.03	0.12	0.89	0.24
PE	86.61	42.92	40.23	3.28	0.26	0.04	0.83	0.26
Alexa Fluor 700	86.15	42.72	40.06	3.00	0.25	0.17	0.06	0.26
Cy7PE	84.49	42.00	38.90	3.09	0.22	0.12	0.79	0.03
Full Panel	82.00	40.88	37.63	3.00	0.26	0.12	0.87	0.30

**Table 10: Fluorescence minus one analysis of the 8-colour panel.** Whole blood from an adult volunteer was stimulated with BCG, and staining performed with the antibodies defined in the table above. The spectral overlap was assessed by investigating the frequencies of cells with or without each single antibody-fluorochrome within the lymphocyte gate. In this analysis, the full 8-colour panel was run, as well as the 8 combinations of the panel minus a single antibody-fluorochrome (shaded boxes). The panel yielded minimal spectral overlap after compensation.

### 3.3.5 Intra-assay coefficient of variation (CV) analysis

Next, we aimed to test if the results generated by our proposed 8-colour panel would be reproducible. Scientifically generated data should be reproducible for it to be considered valid. The CV, computed by dividing the mean by the standard deviation, can be used to assess for variability in a measurement. Although a CV of <8% is ideal for a clinical laboratory test, it is not unusual to find much higher CVs in immunological assays; a CV of up to 30% may be considered acceptable (189). BCG-stimulated whole blood from an adult participant was split into six aliquots and each of the aliquots stained separately in one experiment using the final panel. Standardization of the stimulation and staining procedures ensured the intra-donor assay variability was independent of these procedures. The intra-assay CV ranged from 1.77% for BCG-specific CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> frequencies to 6.98% for BCG-specific CD4<sup>+</sup>IL-2<sup>+</sup> frequencies (**Table 11**). We concluded that the proposed panel would allow for reproducible measurement of the selected functional markers.



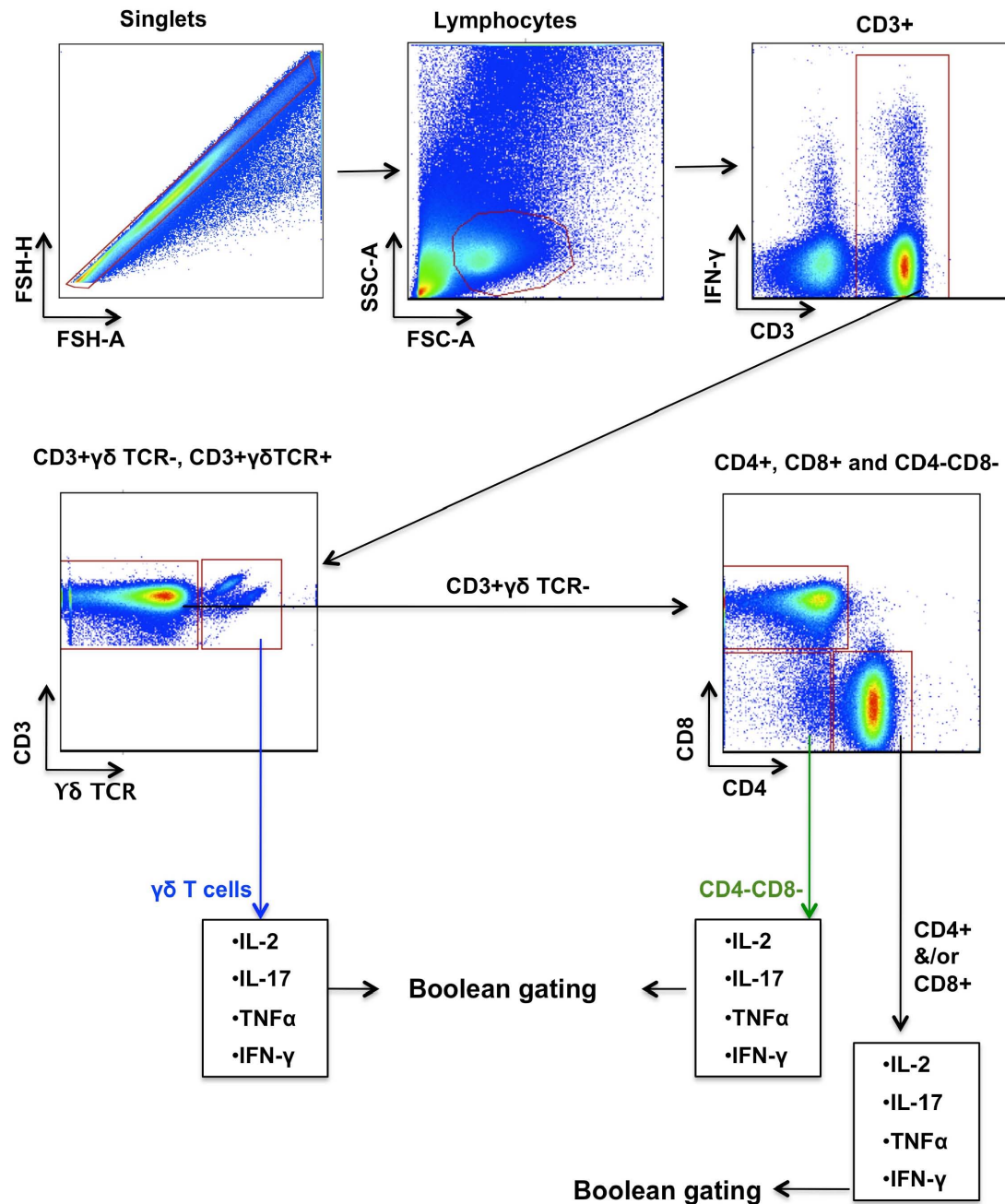
CD4+ Total cytokine expression (%)					CD8+
	IFN- $\gamma$	IL-2	IL-17	TNF- $\alpha$	IFN- $\gamma$
Sample 1	2.15	0.25	0.46	0.31	0.86
Sample 2	2.23	0.30	0.44	0.35	0.84
Sample 3	2.16	0.30	0.47	0.32	0.82
Sample 4	2.21	0.31	0.45	0.32	0.90
Sample 5	2.15	0.30	0.41	0.32	0.85
Sample 6	2.23	0.27	0.46	0.31	0.92
Median	2.19	0.30	0.46	0.32	0.85
Minimum	2.15	0.25	0.41	0.31	0.82
Maximum	2.23	0.31	0.47	0.36	0.92
Mean	2.19	0.29	0.45	0.33	0.87
SD	0.04	0.02	0.02	0.02	0.04
CV (%)	1.77	6.98	4.50	4.75	4.25

**Table 11: Intra-assay coefficient of variation.** Six whole blood samples derived from an adult volunteer were unstimulated or BCG stimulated, stained with the 8-colour panel, and acquired in a single experiment. The medians for the BCG-specific responses are reported, along with the minimums, maximums, and CVs for each cytokine. Background values were subtracted from the respective BCG-specific value.

### 3.3.6 Gating strategy

Our next step was to develop a flow data analysis strategy to address our research questions. The large amount of data generated by large PFC panels can be overwhelming. Thus, a gating strategy must be defined during the panel development process to facilitate a focused and objective driven analysis. Such a strategy is guided by the research questions developed prior to the selection of antigens for the panel. We used the intra-assay CV experiment to develop the analysis strategy. We selected for singlet cells first, followed by lymphocytes. We then gated on CD3<sup>+</sup> T cells followed by CD3<sup>+</sup> $\gamma\delta$ TCR<sup>+</sup> and CD3<sup>+</sup> $\gamma\delta$ TCR<sup>-</sup> cells. The CD4<sup>+</sup> and CD8<sup>+</sup> T cells were then selected from CD3<sup>+</sup> $\gamma\delta$ TCR<sup>-</sup> gate. The BCG-induced IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and

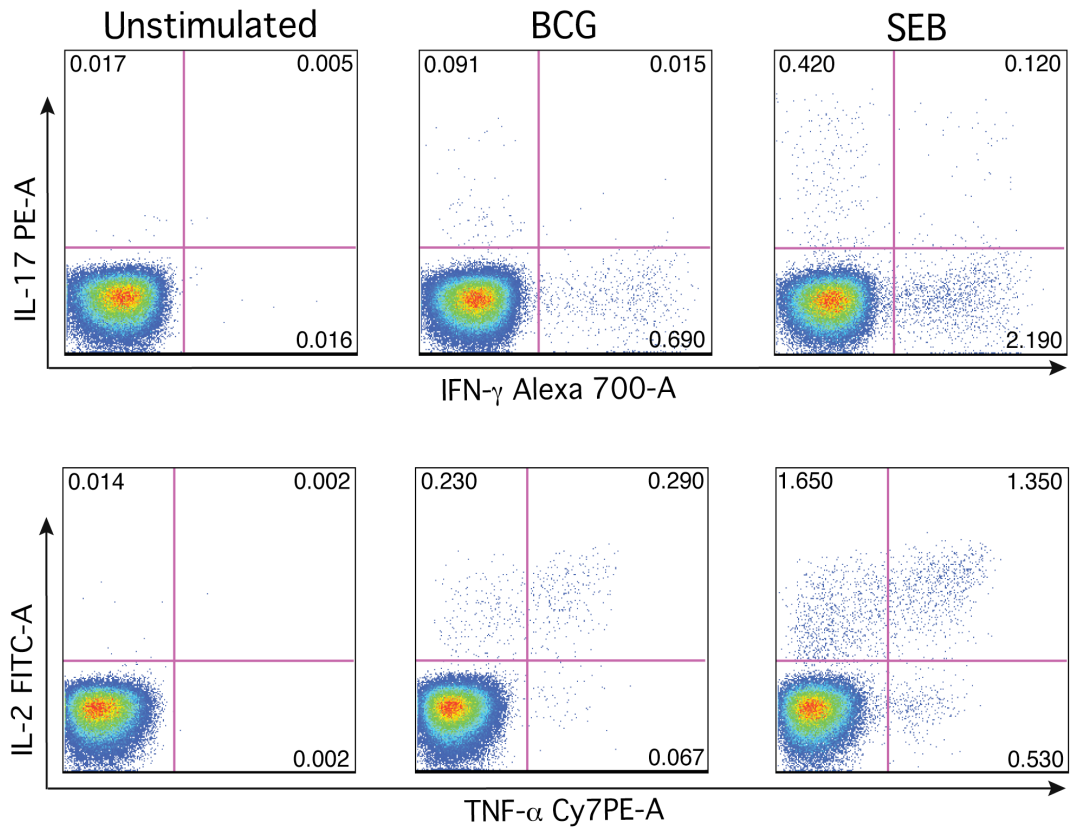
IL-17 cytokine expression was then analyzed for  $\gamma\delta\text{TCR}^+$ ,  $\text{CD4}^+$ ,  $\text{CD8}^+$  and  $\gamma\delta\text{TCR}^-\text{CD4}^-\text{CD8}^-$  T cells (**Figure 23**). This gating strategy was saved as an analysis template. Later, samples were stained, acquired and uploaded on to the analysis template for batch analysis.



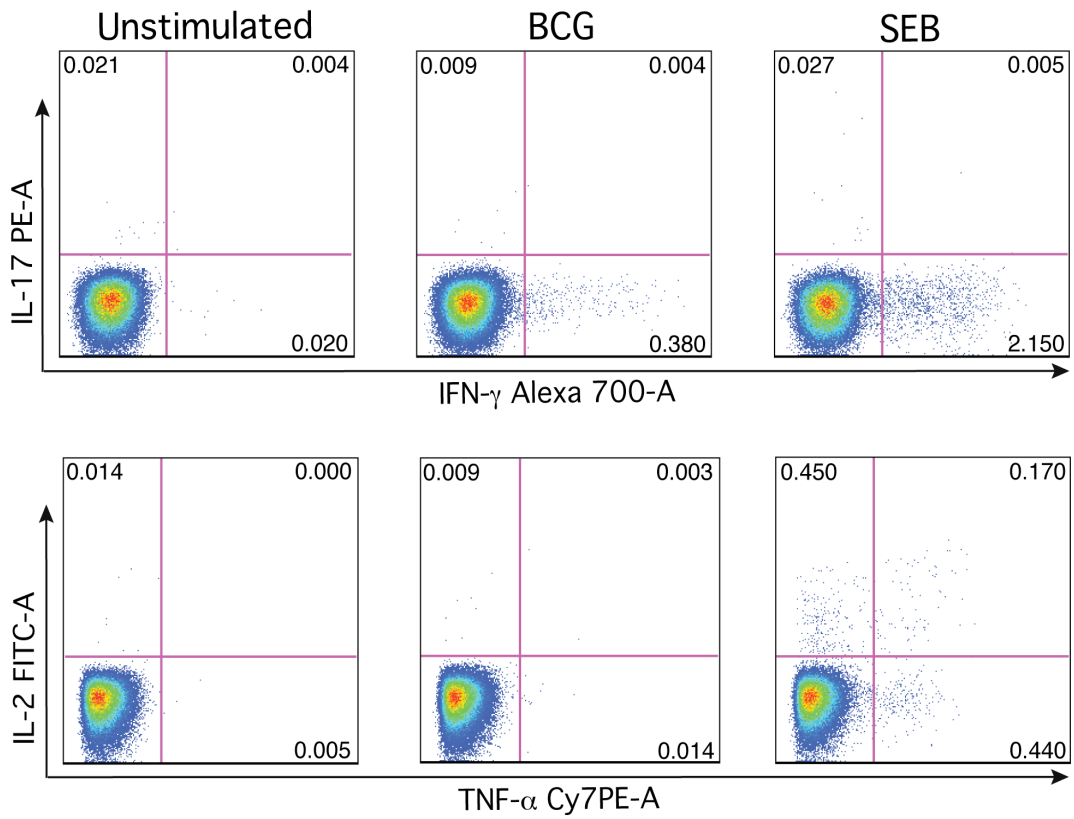
**Figure 23: Gating strategy used for flow cytometry analysis.** Doublets are excluded from the analysis by gating on forward-scatter (FSC)-height vs. FSC-area. The T cells are selected for by gating on  $\text{CD3}^+$  cells. The T cells are subsequently differentiated into  $\text{CD3}^+ \gamma\delta\text{TCR}^-$  and  $\text{CD3}^+ \gamma\delta\text{TCR}^+$ . The  $\text{CD3}^+ \gamma\delta\text{TCR}^-$  population is subdivided into  $\text{CD4}^+$ ,  $\text{CD8}^+$  and  $\text{CD4}^-\text{CD8}^-$  T cells. The intracellular cytokine expression is analyzed as follows: (i) total IFN- $\gamma$ , -IL-2, -IL-17 and -TNF- $\alpha$  expression is first assessed (4x4 outcomes), (ii) and then combinatorial cytokine profiles are assessed after Boolean analysis (4x 16 outcomes).

### 3.3.7 Pilot studies with samples from healthy adult volunteers

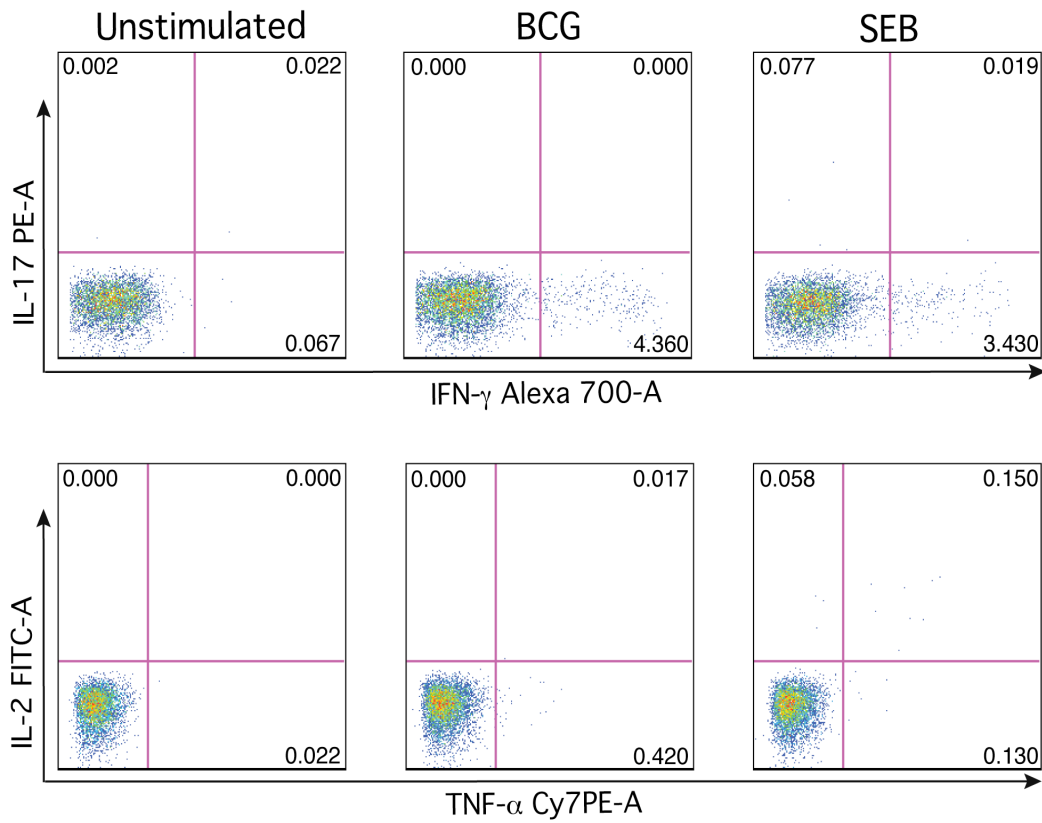
Next, using our optimized 8-colour PFC panel, we aimed to assess whether the patterns of expression of BCG-induced cytokines were comparable to those previously reported in our laboratory. We first analyzed whole blood collected from six adult volunteers. Whole blood was left unstimulated or stimulated with BCG or SEB, followed by ICS analysis. We used the gating strategy described above to analyze the frequencies of T cells expressing cytokines. Data generated from flow analysis was exported to Microsoft Excel and thereafter to GraphPad Prism version 5.0a for analysis. Representative plots showing background, BCG- and SEB-induced IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and IL-17 frequencies in CD4<sup>+</sup> (**Figure 24**), CD8<sup>+</sup> (**Figure 25**),  $\gamma\delta$ TCR<sup>+</sup> (**Figure 26**) and  $\gamma\delta$ TCR<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cells (**Figure 27**) are shown. The BCG-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell co-expression showed that the most dominant subset was single IFN- $\gamma$ -producing cells (**Figure 28A and B**). For CD4<sup>+</sup> T cells, other relevant subsets included polyfunctionals (IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>+</sup>) and bifunctionals (IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>) (**Figure 28A**). All background cytokine expression levels were minimal, allowing for sensitive detection of cytokines. We concluded that the patterns of BCG-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell cytokines were comparable to those previously reported (130).



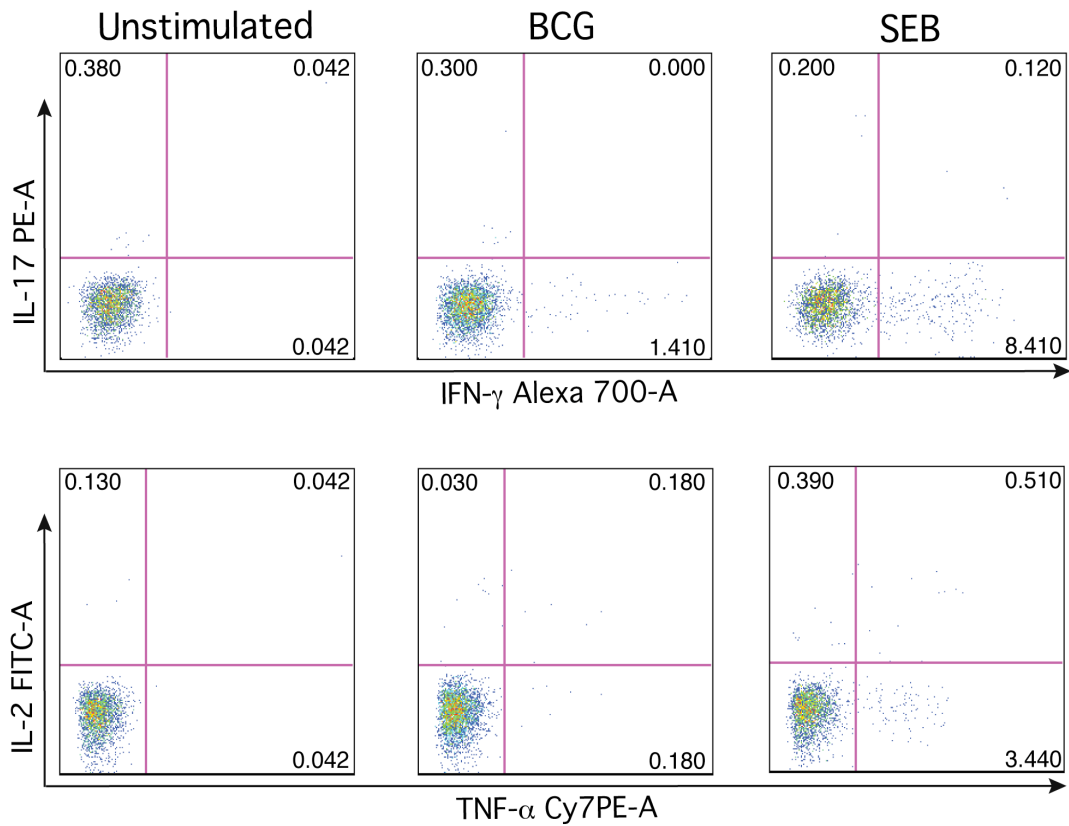
**Figure 24: Intracellular cytokine expression in CD4<sup>+</sup> T cells derived from whole blood of a representative BCG vaccinated adult.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD4<sup>+</sup> T cells were stained intracellularly for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel).



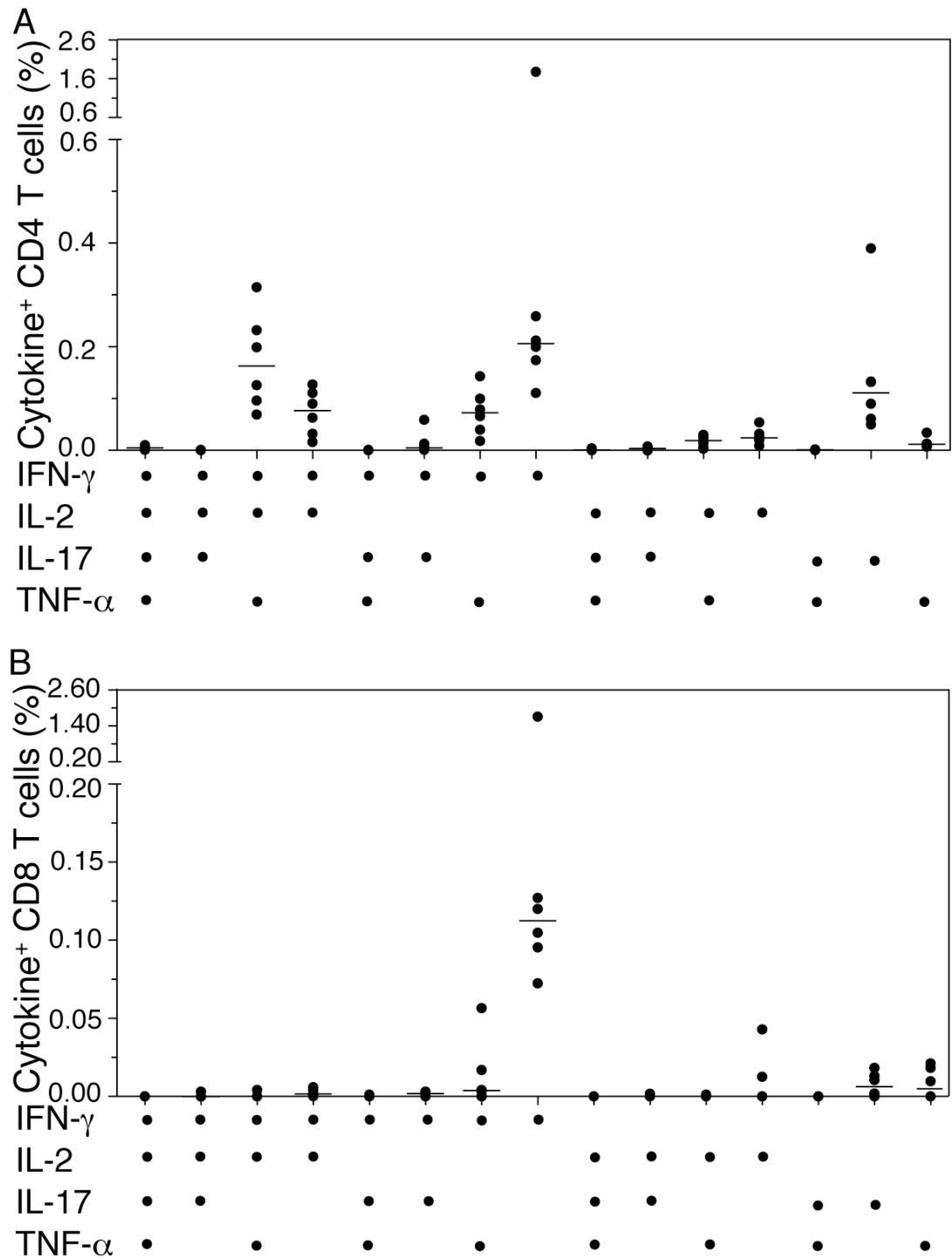
**Figure 25: Intracellular cytokine expression in CD8<sup>+</sup> T cells derived from whole blood of a representative BCG vaccinated adult.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD8<sup>+</sup> T cells were stained intracellularly for IFN-γ and IL-17 (upper panel), TNF-α and IL-2 (lower panel).



**Figure 26: Intracellular cytokine expression in  $\gamma\delta\text{TCR}^+$  cells derived from whole blood of a representative BCG vaccinated adult.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry.  $\gamma\delta\text{T}$  cells were stained intracellularly for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel).



**Figure 27: Intracellular cytokine expression in CD4<sup>+</sup>CD8<sup>+</sup>γδTCR<sup>+</sup> T cells derived from whole blood of a BCG vaccinated adult.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours, and Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD4<sup>+</sup>CD8<sup>+</sup>γδTCR<sup>+</sup> T cells were evaluated for IFN-γ and IL-17 (upper panel), TNF-α and IL-2 (lower panel).

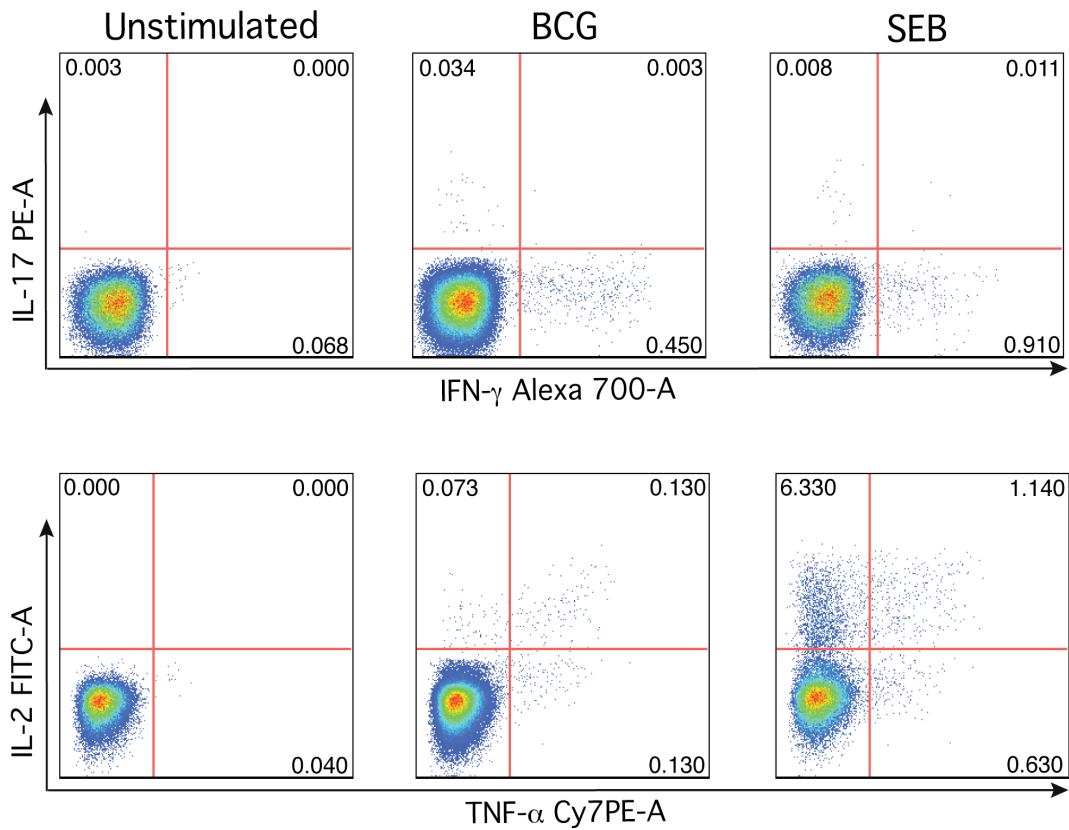


**Figure 28: T cell cytokine expression profiles defined after BCG stimulation of whole blood derived from adult volunteers vaccinated with BCG at birth.** Whole blood samples derived from 6 adult volunteers were left unstimulated or stimulated with BCG for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines and subsequently stained with the 8-colour panel before acquisition on the flow cytometer. Frequencies of cytokine expressing **(A)** CD4<sup>+</sup> T cell subsets and **(B)** CD8<sup>+</sup> T cell subsets are depicted. The lines shown in the graph represent median frequencies.

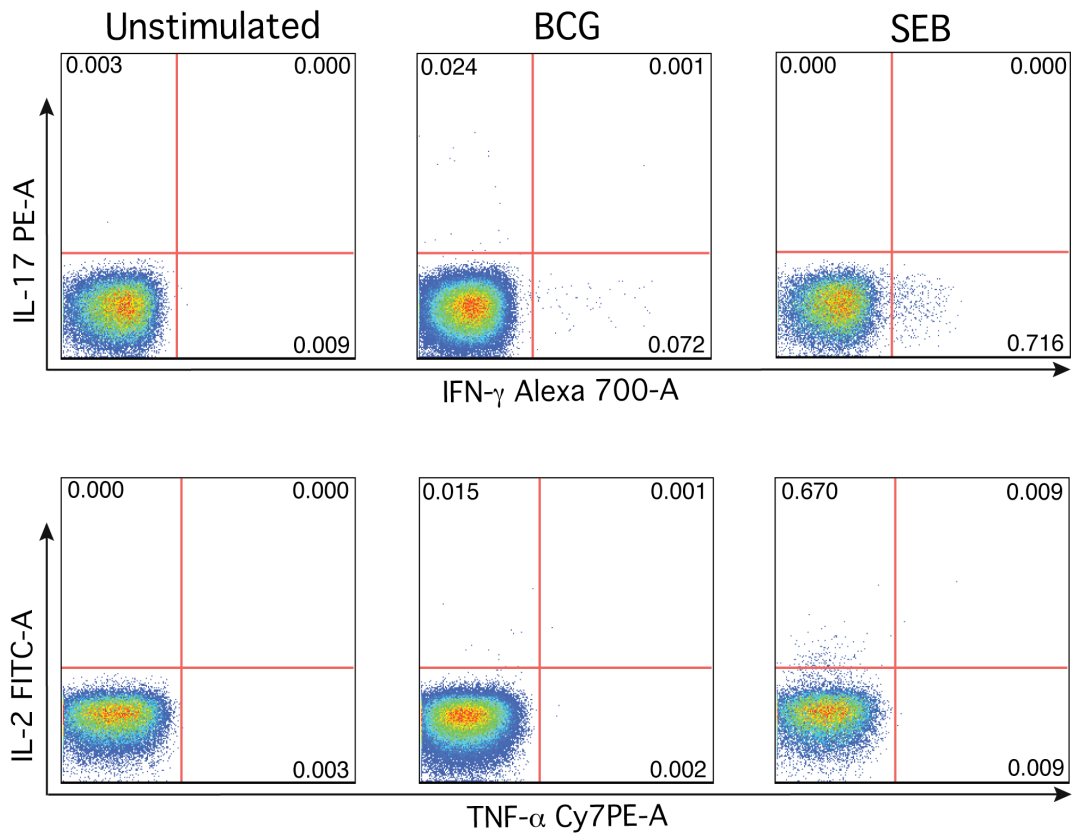


### 3.3.8 Pilot studies with samples from healthy 10-week old infants

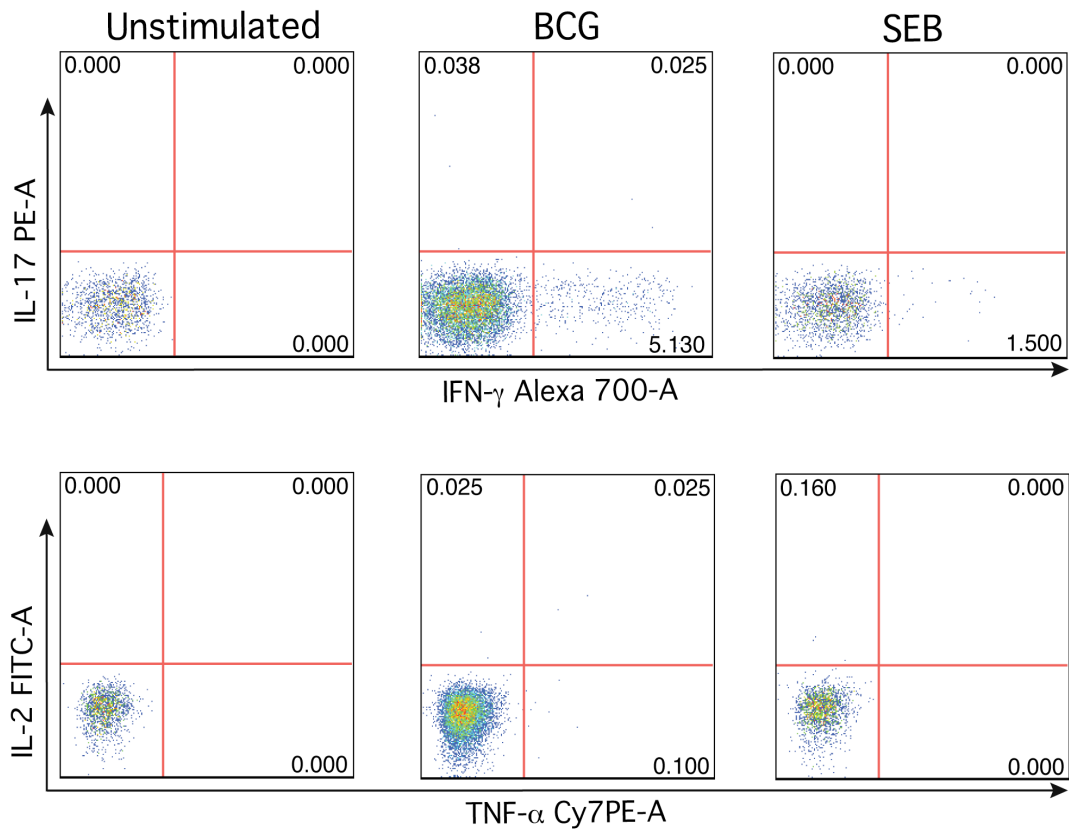
Our next step aimed to assess whether BCG-induced T cell cytokine patterns, measured with our optimized panel, would yield results comparable to those previously reported in a similar assay system with infant-derived whole blood samples. Whole blood from nine infants was left unstimulated or stimulated with BCG or SEB, followed by ICS analysis. We used the gating strategy described above to analyze the frequencies of T cells expressing cytokines. Data generated from flow analysis was exported to Microsoft Excel and thereafter to GraphPad Prism version 5.0a for analysis. Representative plots showing background, BCG and SEB-induced IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and IL-17 frequencies in CD4<sup>+</sup> (**Figure 29**), CD8<sup>+</sup> (**Figure 30**),  $\gamma\delta$ TCR<sup>+</sup> (**Figure 31**) and  $\gamma\delta$ TCR<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cells (**Figure 32**) are shown. The BCG-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell co-expression showed that the most dominant subset was single IFN- $\gamma$ <sup>+</sup> producers (**Figure 33A and B**). For CD4<sup>+</sup> T cells, the other relevant subsets included polyfunctionals (IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>+</sup>) and bifunctionals (IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>) (**Figure 33A**). The cytokine expression patterns observed in infants for CD4<sup>+</sup> and CD8<sup>+</sup> T cells were similar to those observed in adults. Infants showed lower BCG-induced T cell cytokine frequencies than adults. All background cytokine expression levels were minimal, allowing for sensitive detection of cytokines. We concluded that the patterns of BCG-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cell cytokines were comparable to what has been previously reported (130).



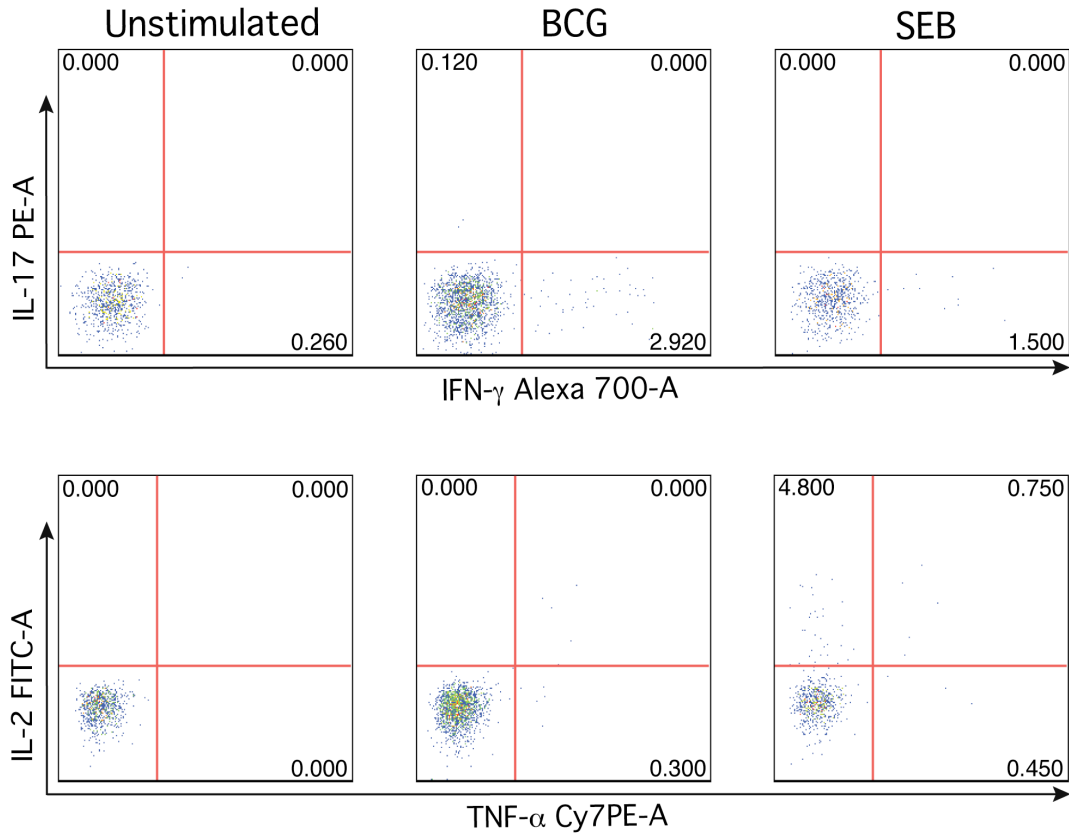
**Figure 29: Intracellular cytokine expression in CD4<sup>+</sup> T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD4<sup>+</sup> T cells were stained intracellularly for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel).



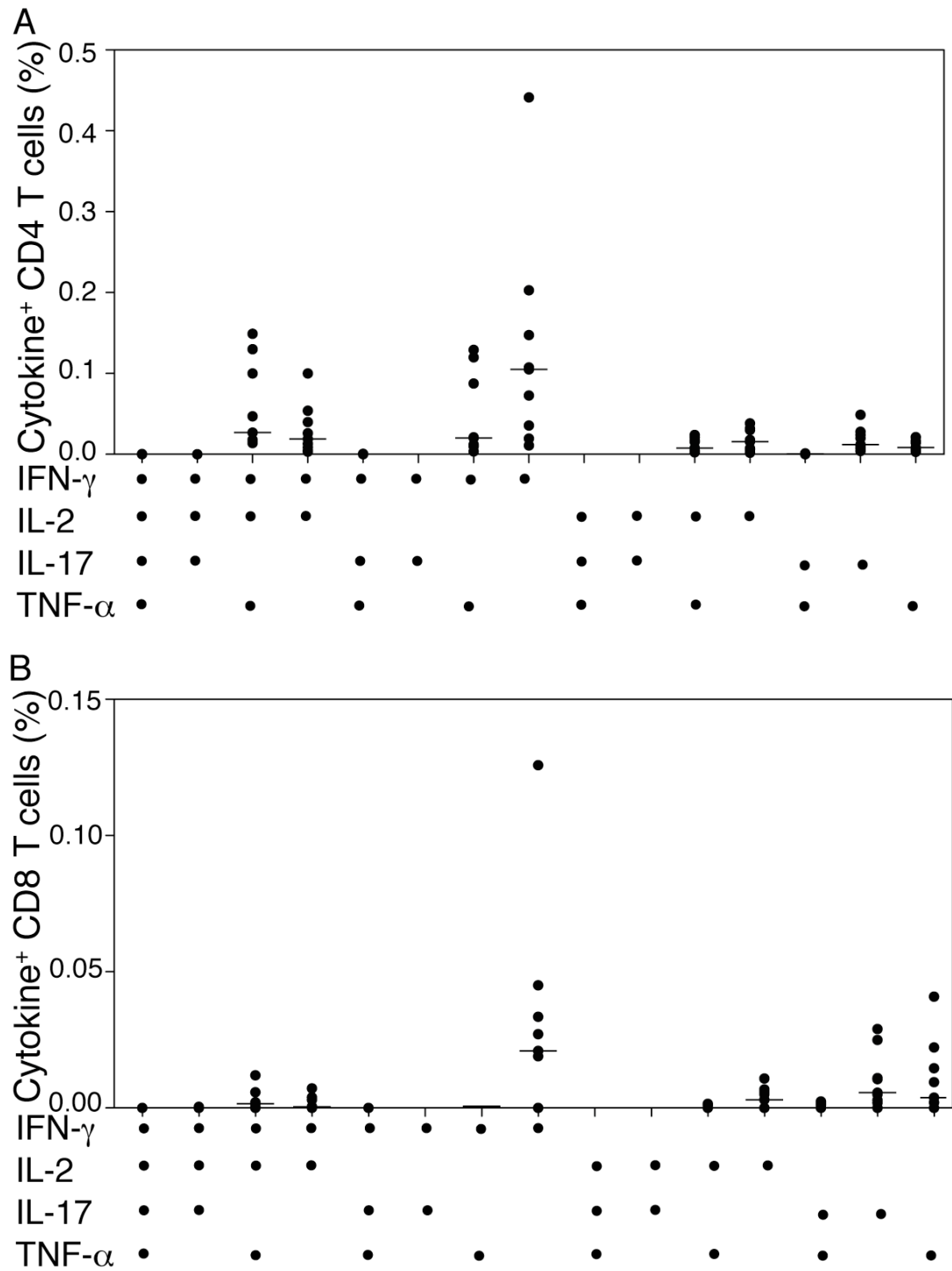
**Figure 30: Intracellular cytokine expression in CD8<sup>+</sup> T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry. CD8<sup>+</sup> T cells were stained intracellularly for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel).



**Figure 31: Intracellular cytokine expression in  $\gamma\delta\text{TCR}^+$  T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry.  $\gamma\delta\text{TCR}^+$  T cells were stained intracellularly for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel). Cytokine expression patterns in the  $\gamma\delta\text{TCR}^+$  subset in infants indicated a predominantly IFN- $\gamma$  expression pattern.



**Figure 32: Intracellular cytokine expression in  $CD4^+CD8^- \gamma\delta TCR^+$  T cells derived from whole blood of a representative 10-week old infant, BCG vaccinated at birth.** Whole blood was either left unstimulated (left panels), or was stimulated with BCG (middle panels) or SEB (right panels) for 12 hours. Brefeldin-A was added in the last 5 hours to capture intracellular cytokines for detection by flow cytometry.  $CD4^+CD8^- \gamma\delta TCR^+$  T cells were evaluated for IFN- $\gamma$  and IL-17 (upper panel), TNF- $\alpha$  and IL-2 (lower panel).



**Figure 33: T cell cytokine expression profiles defined after BCG stimulation of whole blood derived from 10-week old infants vaccinated with BCG at birth.** Whole blood from 9 infants were left unstimulated or stimulated with BCG, and subsequently stained with the optimized 8-colour panel before acquisition. Frequencies of cytokine expressing CD4<sup>+</sup> T cell subsets (A) and CD8<sup>+</sup> T cell subsets (B) are shown. The lines shown in the graph represent median frequencies.

### 3.3.9 Enumeration of T cell events in whole blood from healthy 10-week old infants

Our next step was to estimate the number of CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ TCR<sup>+</sup> and CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> $\gamma\delta$ TCR<sup>-</sup> T cells from one milliliter of stimulated and fixed whole blood cells collected from a 10-week old infant. Adequate cell numbers are critical for reliable PFC analysis. Therefore it was important to establish whether we had sufficient cells for analysis. We used data generated from our nine infants' pilot studies. The median number of cells for CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ TCR<sup>+</sup>, and CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> $\gamma\delta$ TCR<sup>-</sup> are provided in **Table 12** below.

Cell subset	Median (minimum-maximum) number of cells
CD4 <sup>+</sup>	157,601 (31,815 - 597,000)
CD8 <sup>+</sup>	56,690 (4,743 - 242,118)
$\gamma\delta$ TCR <sup>+</sup>	9,972 (1,509 - 27,721)
CD3 <sup>+</sup> CD4 <sup>-</sup> CD8 <sup>-</sup> $\gamma\delta$ TCR <sup>-</sup>	6,127 (2,001- 48,789)

**Table 12. Expected cell yields per milliliter of 10-week old infant blood.** For the proposed studies, infant whole blood was cryopreserved in aliquots of 0.5mL for the unstimulated samples (negative controls), 1mL for the BCG stimulated samples, and 0.5mL for the SEB samples (positive controls). This table shows the average yields of lymphocytes calculated from 3-pooled aliquots (Unstimulated, BCG and SEB) of whole blood from 9 individual infants.

### 3.3.10 Assessment of minimum T cell numbers required for reliable analysis of samples from healthy 10-week old infants

Finally, we aimed to determine the minimum number of cells that should be acquired to allow for reliable analysis of BCG-induced T cell cytokine expression in cryopreserved infant blood samples. In flow cytometry, collection of few cellular events increases the error rate of the results. We utilized an algorithm developed by Dr Holden Maecker ([http://maeckerlab.typepad.com/maeckerlab\\_weblog/maeckerlab\\_protocols\\_and\\_tools/index.html](http://maeckerlab.typepad.com/maeckerlab_weblog/maeckerlab_protocols_and_tools/index.html) accessed on 16/10/2007). The algorithm factors in the background levels detected in each channel and the respective positive

levels, to determine the minimum number of events required for reliable analysis. We used the data generated from the nine infants in our pilot experiments, which revealed that approximately, 120,000 CD4<sup>+</sup> T cells are required to obtain statistically significant results over background for all cytokines. This cell count was based on BCG-induced IL-17, which was least expressed of all the cytokines measured. For CD8<sup>+</sup> T cells, we only established the minimum event count (28,649) for the IFN- $\gamma$ <sup>+</sup> subset, which was demonstrated to be the most dominant subset (**Table 13**). We concluded that we had sufficient cells in the cryopreserved infant samples for our analysis. Nevertheless, we planned to acquire the maximum number of cells in our studies.

	<b>CD4+</b>				<b>CD8+</b>
Cytokine Expression	IL-2+	IL-17+	TNF+	IFN- $\gamma$ +	IFN- $\gamma$ +
Background (%)	0.009	0.011	0.002	0.015	0.030
BCG response (%)	0.073	0.028	0.050	0.191	0.090
<b>Minimum number of cells required</b>	<b>17,210</b>	<b>116,033</b>	<b>19,405</b>	<b>5,713</b>	<b>28,649</b>

**Table 13. Power calculation for event acquisition.** The median levels for backgrounds and BCG responses for each cytokine were calculated from a whole blood experiment performed on 9 infants.  
([http://maeckerlab.typepad.com/maeckerlab\\_weblog/maeckerlab\\_protocols\\_and\\_tools/index.html](http://maeckerlab.typepad.com/maeckerlab_weblog/maeckerlab_protocols_and_tools/index.html)) (accessed on 16/10/2007).

### 3.4 Discussion

In this chapter, we report the successful optimization of an 8-colour PFC panel. We used the optimized panel to measure the complex BCG-specific T cell cytokine pattern in adult and infant stimulated whole blood cells. We established that cryopreserved samples, collected for the study would yield sufficient cells to allow for a reliable analysis of BCG-specific T cell subsets.

An optimal PFC panel must be preceded by clearly defined research questions, from which a list of candidate markers is developed. Our candidate markers (CD3, CD4, CD8,  $\gamma\delta$ T, IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-13, IL-17



and IL-22) were selected based on the roles they are presumed to play in anti-*Mtb* host immunity. Subsequent exclusion of candidate markers was conducted after careful evaluation, and the following parameters were considered for selection of the panel: (i) the relevance of markers to address the research questions was determined through extensive literature reviews. (ii) the expression levels of the selected markers were assessed using the well-defined assay system. (iii) the capacity of the flow cytometer to measure multiple parameters was assessed. (iv) the quality and availability of antibody-fluorochrome combinations was carefully considered. (v) the availability of PFC panels already optimized and available in our laboratory. All these parameters were considered during the successful development of the panel.

Optimal PMT settings must be established prior to assessing the performance of the panel. Suboptimal voltages may result in poor compensation between the different channels, and thereby compromise the outcome. The FMO for our optimized 8-colour PFC panel revealed minimal spectral “spill over” into any of the channels utilized. This finding, in addition to the low intra-assay CVs (2-7%) for the panel indicates the reliability of the results generated.

We found our cytometer could reliably measure the BCG-induced T cell cytokines IFN- $\gamma$  and IL-2, although the limit of detection for these cytokines were not as low as those of TNF- $\alpha$  and IL-17. We suspect this minor difference in detection limit may be due to the dim fluorochromes conjugated to IFN- $\gamma$  and IL-2 cytokines. Staining index, a measure of the brightness of a fluorochrome, may vary with antibody-fluorochrome combinations (195) and possibly affect the limit of detection. Nevertheless, in our assay system, BCG-induced IFN- $\gamma$ , and IL-2 are highly expressed cytokines. Hence, staining of these cytokines with fluorochromes of lower brightness index score (Alexa Fluor 700 and FITC, respectively) was sufficient to allow for sensitive measurement.

When optimizing panels for application in paediatric settings where blood volumes are limited, we emphasize the importance of using adult blood samples during the preliminary optimization steps. Adult blood samples are generally easier to obtain and larger volumes are available for troubleshooting. Nevertheless, before the analysis of infant samples, the panels optimized using adult samples must be tested with infant samples that closely resemble the actual study samples. We followed this process to generate results from whole blood derived from 9 infants that had been stimulated, processed and cryopreserved for a long period of time. Importantly, our results from the infant samples was comparable in magnitude and patterns to those reported by Soares *et al.* (130).

Our data, and previous data from our lab, show that the detectable specific response in fixed cells from BCG stimulated whole blood is higher when using adult, compared with infant samples. This may be, in part, because infants have higher numbers of absolute lymphocytes count compared with adults (220), which can reduce the frequencies of the specific cells in infants. Also, it could be because infants have lower frequencies of memory lymphocytes than adults (220). Our approach did not include an assessment of absolute cell counts, nor memory markers. We therefore could not correct for these variables when comparing the detectable specific response in infant and adult samples.

The challenges of conducting large TB immune correlates of risk studies in pediatric settings are multiple: (i) limited blood volumes are available, (ii) standardization of assay procedures, (iii) prolonged cryopreservation of samples, (iv) optimization of cytometry panels, and (v) development of optimal data analysis procedures. In this chapter, we show an iterative process for successfully overcoming these challenges and for generating reliable results. Importantly, from this optimization process, QDot 605-conjugated antibody was introduced as a useful and reliable reagent in our laboratory, and a 6-colour flow panel was standardised that remains a core platform in our laboratory.

The importance of optimization in the development of flow cytometry panels is well recognized (195). Not only does this process ensure that reliable results are generated, but also yields a solid foundation for future studies. We utilized the panel described here in (Chapter four) and a modified version (Chapter five) to generate results of significant importance to the field of TB vaccinology.

### **3.5 Contributions**

B.M.N. Kagina designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B. Abel, and other members of the SATVI laboratory.

## **Chapter 4: Specific T cell Frequency and Cytokine Expression Profile Do Not Correlate with Risk of Developing Tuberculosis, Following BCG Vaccination of Newborns**

**Authors:** Benjamin M. N. Kagina, Brian Abel, Thomas J. Scriba, Elizabeth J. Hughes, Alana Keyser, Andreia Soares, Hoyam Gamiieldien, Mzwandile Sidibana, Mark Hatherill, Sebastian Gelderbloem, Hassan Mahomed, Anthony Hawkridge, Gregory Hussey, Gilla Kaplan, Willem A. Hanekom, and other members of SATVI

**Studies reported in this chapter were published in the American Journal of Respiratory and Critical Care Medicine (*Am J Respir Crit Care Med.* 2010 Oct 15;182(8):1073-9. Epub 2010 Jun 17). Additional results that were not published are included.**

### **4.1 Abstract**

The development of more effective TB vaccines is considered a key intervention in the control of global TB pandemic. However, the lack of known immune correlates of protection against TB is hampering development and testing of new vaccines against the disease. Immunogenicity of new TB vaccines is commonly assessed by measuring the frequency and cytokine expression profile of T cells. We tested whether this outcome correlates with risk of developing childhood TB disease, following newborn vaccination with BCG.

Whole blood from 10-week old infants, routinely vaccinated with BCG at birth, was incubated with BCG for 12 hours, followed by cryopreservation for intracellular cytokine analysis. Infants were followed for 2 years to identify those who developed culture-positive TB – these infants were regarded as TB cases. Infants who did not develop TB disease despite exposure to TB in the household, and another group of randomly selected infants who were never evaluated for TB, were also identified – these groups were regarded as TB controls. Cells from these groups were thawed, and using PFC, T cell-

specific expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-17 in CD4, CD8, and  $\gamma\delta$  was measured.

5,662 infants were enrolled; 29 cases and 2 groups of 55 control infants were identified. There was no difference in frequencies of BCG-specific CD4, CD8 and  $\gamma\delta$  T cells between the 3 groups of infants. Although BCG induced complex patterns of intracellular cytokine expression, there were no differences between cases and control infants.

The frequency and cytokine profile of mycobacteria-specific T cells did not correlate with risk of developing childhood TB. We propose that critical components of immunity against *M.tb*, such as CD4 T cell IFN- $\gamma$  production, may not necessarily translate into immune correlates of protection against TB disease.

#### **4.2 Introduction**

TB is a disease of global public health concern. In 2008, the WHO reported that TB disease kills 1.7 million people worldwide each year (128). The current TB vaccine, BCG, affords ~80% protection against severe forms of childhood TB (221, 222). However, BCG's protection against pulmonary TB, particularly in adults, is highly variable and mostly poor (50). Adults with lung TB spread the disease; new, better TB vaccines that target pulmonary disease are therefore needed urgently.

Our knowledge of immune correlates of protection against TB remains incomplete. Consequently, assessment of immunogenicity of TB vaccines may at best be a measure of vaccine take. Current evaluations of vaccine take focuses on immunity essential for protection against TB. For example, experimental and clinical evidence support a critical role for CD4 T cells, particularly IFN- $\gamma$  production by these cells in protection against TB (223, 224). IFN- $\gamma$  production is therefore the most commonly measured when determining TB vaccine take. Since important roles for other type 1 cytokines, such as TNF- $\alpha$  and IL-2 (210, 225, 226), and for CD8 T cells

(227-229), in protection against TB have also been described, all these markers are commonly measured together, using PFC after short-term stimulation of whole blood or PBMC (105, 106, 230-233). Experimental animal studies assessing the efficacy of novel TB vaccines have reported an association between presence of mycobacteria-specific polyfunctional T cells that co-express IFN- $\gamma$ , TNF- $\alpha$  and IL-2 at the site of the infection, and protection against TB (234). These findings have stimulated much interest in evaluating this subset of T cells in clinical trials.

The study in this chapter aimed to assess whether these markers correlate with risk of developing childhood TB, following newborn vaccination with BCG. We complemented this assessment by also determining whether expression of IL-17 correlates with risk of developing childhood TB disease. Memory Th17 cells are present in peripheral blood of persons exposed to mycobacteria (212); experimental evidence supports a role for these cells to induce chemokine release in the lung resulting in Th1 cell recruitment (214). Furthermore, the magnitude of IL-17 response has been shown to correlate with the clinical outcome of *M.tb* infection (114). We also wished to determine whether  $\gamma\delta$  T cell activation correlates with a reduced risk of TB disease. This was based on potent mycobacteria-specific activation of  $\gamma\delta$  T cells in 7 month-old infants who had received BCG vaccine at birth (235), and on experimental evidence that suggests an important role in protection against TB, possibly by activating APC to prime Th1 responses (200).

## **4.3 Materials and methods**

### **4.3.1 Participant enrollment**

We enrolled participants at the SATVI field site in the Worcester area, near Cape Town, South Africa. This area has one of the highest TB incidence rates in the world, documented to be in excess of 1,000/100,000/year in children <2 years of age (5). The study was nested within a randomized controlled trial (RCT) (101), which aimed to determine whether intradermal or percutaneous delivery of Japanese BCG at birth resulted in equivalent protection against TB. The following were exclusion criteria at 10 weeks of

age: mother known to be HIV-infected, BCG not received by infant within 24 hours of birth, significant perinatal complications in the infant, any acute or chronic disease in the infant at the time of enrollment, clinically apparent anemia in the infant, and household contact with any person with TB disease, or any person who was coughing. The study was conducted according to the US Department of Health and Human Services and Good Clinical Practice guidelines. This included protocol approval by the University of Cape Town Research Ethics Committee and written informed consent from the parent or legal guardian.

#### **4.3.2 Blood collection, stimulation and cryopreservation**

At 10 weeks of age, heparinized blood was collected from all infants. 1mL was immediately incubated with BCG (SSI strain,  $1.2 \times 10^6$  organisms/mL), as previously described (178). Medium alone served as negative control, while SEB (Sigma-Aldrich, 10µg/mL) was used as positive control. To all conditions the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences, 1µg/mL each) were added, as this results in enhancement of specific responses (178). Blood was incubated for 7 hours at 37°C. Brefeldin-A was then added, followed by incubation for an additional 5 hours. Cells were then harvested, fixed and cryopreserved as previously described (178).

#### **4.3.3 Participant follow up and evaluation**

Participants were followed for 2 years. Community-wide passive surveillance systems identified patients with TB disease and children with symptoms suggestive of TB disease, or from households where an adult had TB disease (101). Children who fell into the latter 2 categories were followed up actively; among these, participants who never developed TB over the 2 year follow up period were classified as household controls, while participants who developed TB disease over this period were classified as TB cases. Community controls were randomly selected from the passive surveillance arm and were never evaluated for TB. The parent study (101), reports the detailed criteria employed to detect all cases of TB disease among participants, up to the age of 2 years. All infants who had symptoms

compatible with TB disease, or who had contact with an adult with TB disease, were admitted to a dedicated research ward for clinical examination, chest radiography, tuberculin skin testing, two early morning gastric aspirations and two sputum inductions for *M.tb* smear and culture (101). All infants admitted to the research ward were also tested for HIV infection: a positive antibody test resulted in exclusion.

#### **4.3.4 Intracellular cytokine staining and multi-parameter flow cytometry**

Cryopreserved cells from the cases and control groups (see data analysis below) were thawed, washed and permeabilised using Perm wash solution (BD Biosciences). Cells were then incubated at 4°C for 1 hour with fluorescence-conjugated antibodies directed against surface antigens and intracellular cytokines. The following antibodies were used: anti-CD3 Pac Blue (clone UCHT1), anti-CD8 Cy5.5PerCP (SK-1), anti- $\gamma\delta$ TCR APC (B1), anti-TNF- $\alpha$  Cy7PE (Mab11), anti-IFN- $\gamma$  Alexa 700 (B27), anti-IL-2 FITC (5344.111, all from BD Biosciences), anti-CD4 QDot605 (S3.5, Invitrogen), and anti-IL-17 PE (eBio64CAP17, eBioscience). Cells were acquired on a LSRII flow cytometer (BD Biosciences) configured with 3 lasers and 10 detectors, using FACS Diva 6.1 software. Optimal photomultiplier tube (PMT) settings were established for this study prior to sample analysis. Cytometer Setting and Tracking (CST) beads (BD Biosciences) were used to record the target MFI values for the baseline settings, and these calibrations were performed each day prior to sample acquisition. Compensation settings were set using anti-mouse kappa-beads (BD Biosciences), labelled with the respective fluorochrome-conjugated antibodies. Flowjo version 8.8.4 (Treestar) was used to compensate and to analyze the flow cytometric data. Boolean gating was applied to generate combinations of cytokine expressing CD4 and CD8 T cell subsets.

#### **4.3.5 Data analysis**

Flow cytometric analysis was compared between the 3 groups of infants: (1) those who ultimately developed culture-confirmed TB disease; regarded as TB cases, (2) those who were evaluated for TB because of household



exposure to an adult with TB disease, but found not to have TB; regarded as household controls, (3) a third, randomly selected group, who had no known exposure to an adult with TB disease, or symptoms compatible with TB disease, and were therefore never evaluated for TB; this was the second group regarded as community controls. Twenty TB cases were excluded from the analysis due to insufficient sample volume for analysis. The microbiological, clinical and radiological features of the excluded TB cases were comparable to those of the included TB cases.

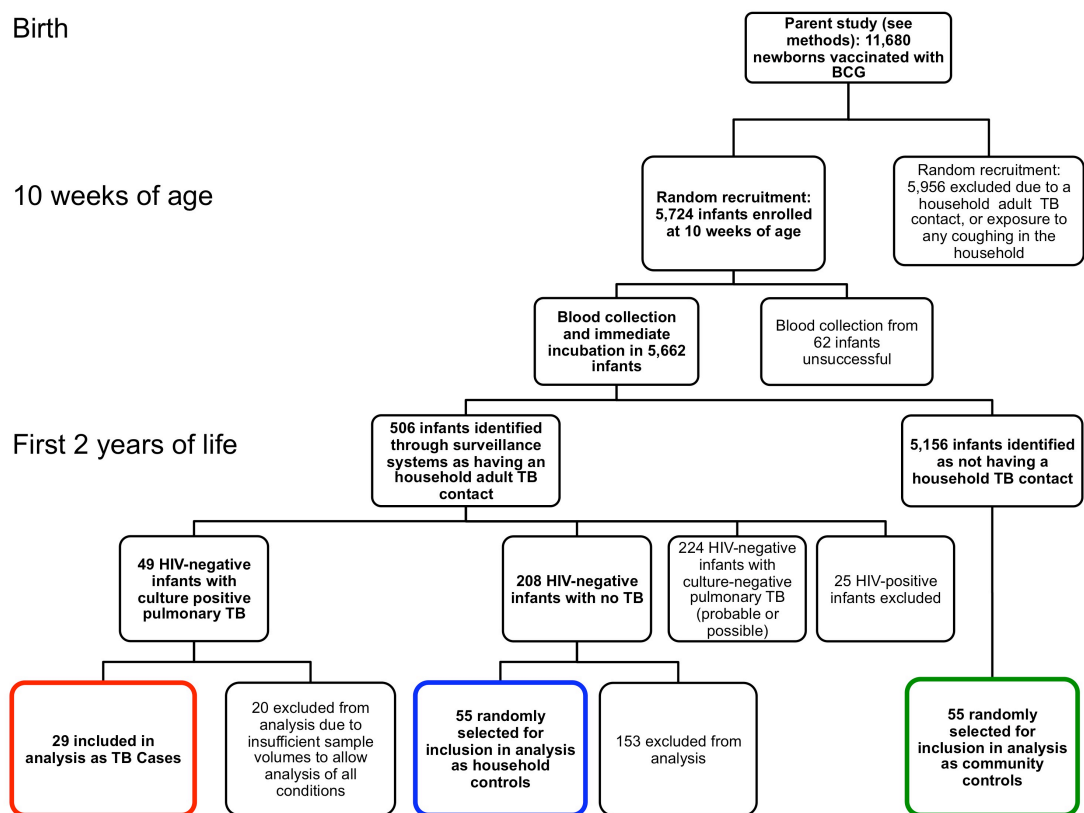
For flow cytometric analysis of cytokine-expressing cells, frequencies of cytokine expression from the negative control, i.e., blood incubated with co-stimulatory antibodies alone, were subtracted from the BCG-specific responses. Participants were excluded from the analysis for any of the following reasons: (1) a positive control (SEB) CD4 T cell response less than the median plus 3 median average deviations (MADs) of the CD4 T cell response in the negative control, (2) CD4 T cell frequencies in the negative control and in the BCG sample in a similar range, suggesting possible contamination of the negative control, (3) number of CD3 T cell events counted < 75,000. In infants, BCG-specific responses are predominantly present in the CD4 T cell subset. As such, all exclusions were based on the responses in this subset.

For the analysis of median fluorescence intensities (MFI) of the BCG-stimulated samples, further exclusions occurred if results from participants did not meet any of the following conditions: (1) ratio of BCG to unstimulated frequencies > 2, (2) frequencies of BCG-specific cells > 0.01%, (3) number of positive events in the BCG-stimulated sample > 20. The Kruskal-Wallis test was used to assess differences between the 3 groups, and when differences had a p value <0.05, a Mann-Whitney U test was used to assess differences between individual groups; p values <0.05 were considered significant.

## 4.4 Results

### 4.4.1 Study participants

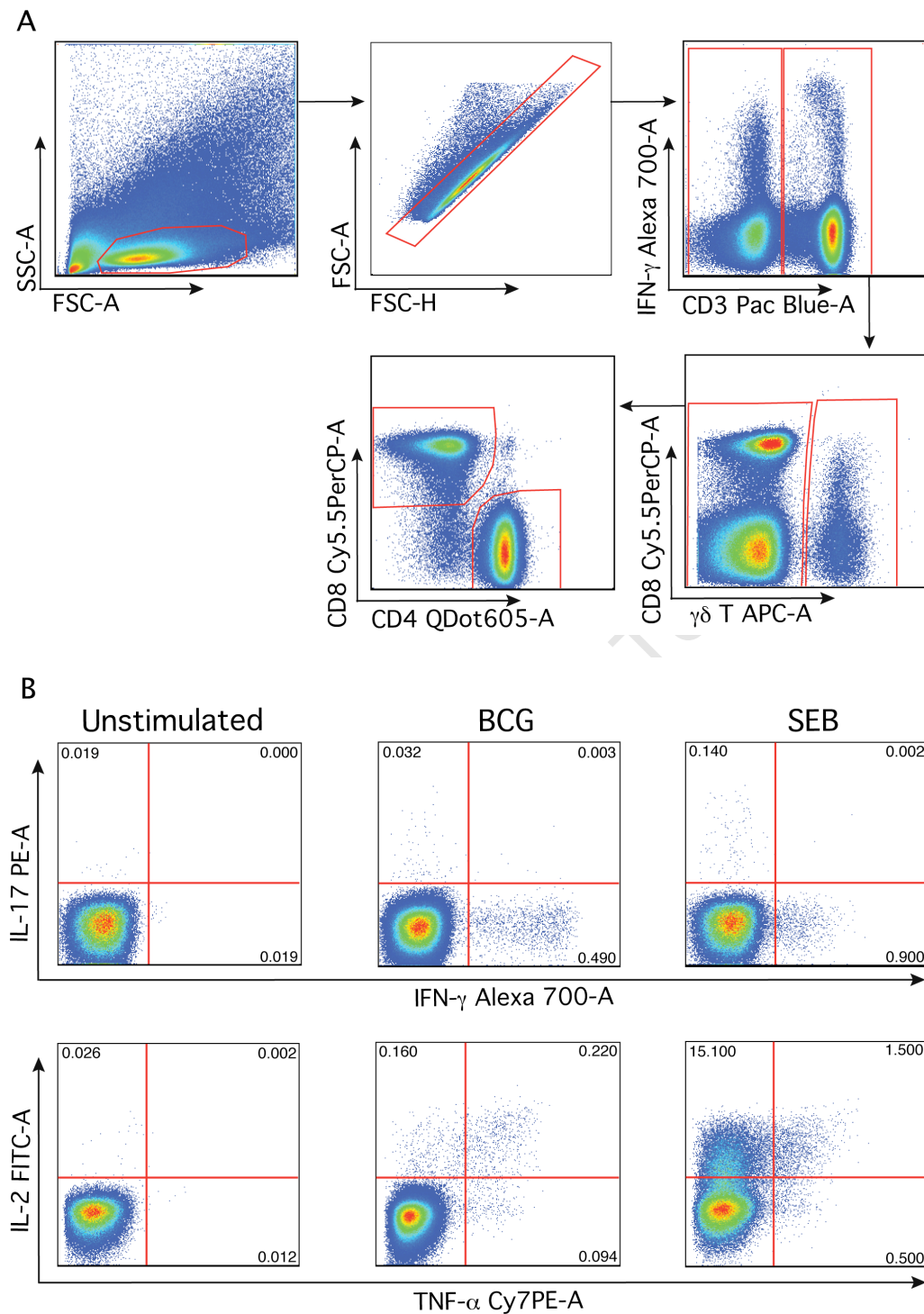
A total of 5,724 infants routinely vaccinated with BCG at birth, were randomly enrolled from the parent cohort of 11,680 infants (101). Identification of the 3 infant groups, with clinical exclusions, is shown in **Figure 34**. All the three infant groups had comparable proportions of infants who received either intradermal or percutaneous BCG at birth.



**Figure 34: Study participants.** Recruitment and enrollment of participants into the study.

### 4.4.2 Gating strategy

To analyse for specific cells of interest, we modified the strategy developed during the optimization process described in chapter three.



**Figure 35: Gating strategy.** Flow cytometric analysis of BCG-induced T cell cytokine production. Whole blood was incubated with BCG for 12 h. Representative dotplots from a single participant are shown. **(A)** Gating strategy used to identify CD4, CD8, and  $\gamma\delta$  T cells. From left to right, leukocytes from whole blood were acquired and cell doublets excluded using forward scatter-area versus forward scatter-height parameters. T cells were identified by assessing CD3 expression against IFN- $\gamma$  expression, which enables inclusion of any T cells that may have down-regulated CD3 expression upon activation. Subsequently, CD3+ T cells were differentiated into conventional T cells, which did not express  $\gamma\delta$ TCR, and  $\gamma\delta$  T cells, by assessing  $\gamma\delta$  expression against CD8 expression. Finally, the conventional T cell population was divided into CD4+ and CD8+ T cells. **(B)** Representative dot plots of cytokine co-expression patterns in CD4 T cells from unstimulated, BCG and SEB-stimulated conditions.

#### 4.4.3 Participants excluded from analysis based on quality control criteria

To ensure only reliable data analysis was reported, we developed an internal quality control system that would exclude participants whose data fell below the quality control criteria as described in the data analysis section. The **Tables 14A to I** show a summary of the number of participants excluded.

**Table 14A: Exclusion of participants from analysis based on flow cytometry data: Frequency of cytokine-producing cells**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
SEB responses < median + 3 MAD of the negative control	0	1	1
Frequency of negative control similar to that of BCG sample	0	0	3
CD3 T cell counts <75,000	0	2	0

**Table 14B: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IL-2 only**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	0	6	5
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	0	0	1

**Table 14C: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IFN- $\gamma$  only**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	2	3	5
BCG-specific frequency < 0.01%	0	0	3
BCG event counts <20	0	0	0

**Table 14D: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing TNF- $\alpha$  only**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	11	18	22
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	1	0	2

**Table 14E: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IL-17 only**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	6	15	21
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	0	0	1

**Table 14F: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IFN- $\gamma$  and IL-2 together**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	1	0	0
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	1	4	3

**Table 14G: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IL-2 and TNF- $\alpha$  together**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	1	1	0
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	2	5	3

**Table 14H: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IFN- $\gamma$  and TNF- $\alpha$  together**

Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	1	2	2
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	1	3	3

**Table 14I: Exclusion of participants from analysis based on flow cytometry data: MFI in cells expressing IFN- $\gamma$ , IL-2 and TNF- $\alpha$  together**

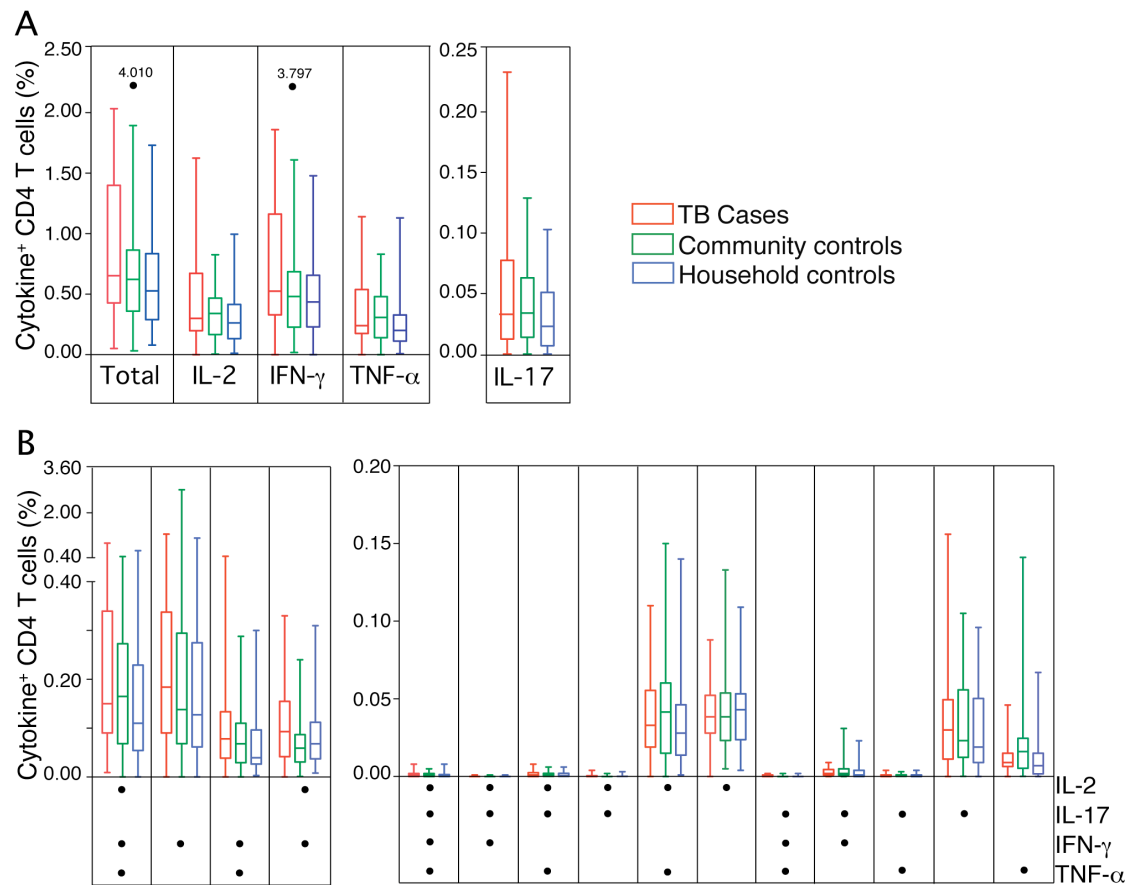
Reason for exclusion	TB cases excluded (n)	Community controls excluded (n)	Household controls excluded (n)
Ratio of BCG to unstimulated frequency <2	1	0	0
BCG-specific frequency < 0.01%	0	0	0
BCG event counts <20	0	3	3

#### 4.4.4 Frequency and cytokine profile of BCG-specific CD4 T cells

To assess whether BCG-specific T cells was associated with the risk of childhood TB disease development, following BCG vaccination at birth, we used a intracellular cytokine assay to evaluate the frequency and cytokine profiles of BCG-specific T cells: A median of 409,077 (154,687 - 802,636) CD3 T cells were evaluated. Results from 7 participants were excluded from analysis, because the inclusion criteria for analysis were not met (**Table 14A**). we compared CD4 T cell expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-17 in the cases and the two control groups.

Frequencies of total cytokine-expressing BCG-specific CD4 T cells, expressing any of the 4 cytokines, were not different in the 3 groups (**Figure 36A**). In addition, no differences were observed when frequencies of BCG-specific Th1 CD4 T cells, either expressing IFN- $\gamma$ , or TNF- $\alpha$ , or IL-2, were compared (**Figure 36A**). IL-17<sup>+</sup>-expressing Th17 cells were also not different

(**Figure 36A**). In all three groups, IFN- $\gamma$ <sup>+</sup>-expressing CD4 T cells comprised the dominant subset (**Figure 36A**), whilst IL-17<sup>+</sup>-expressing CD4 T cells was the least frequent (**Figure 36A**). We also compared the cytokine co-expression pattern of BCG-specific CD4 T cells. Polyfunctional CD4 T cells, co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, and single IFN- $\gamma$ <sup>+</sup>-expressing CD4 T cells were the dominant subsets (**Figure 36B**). No differences were observed in the frequencies of any BCG-specific CD4 T cells subset within the three groups including polyfunctional CD4 T cells and single IFN- $\gamma$ <sup>+</sup> expressing CD4 T cells. We conclude that using this assay system; both quantitative and qualitative BCG-specific CD4 T cell responses are not associated with reduced risk of TB disease development in infants, following BCG vaccination at birth.



**Figure 36: Frequency and cytokine expression profile of BCG-specific CD4 T cells**  
**(A)** Frequencies of total, IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and IL-17 cytokine expressing CD4 T cells, as detected by an intracellular cytokine assay, following incubation of whole blood with BCG. Total cytokine frequencies incorporate all cytokine positive CD4 T cells. Results are shown in box and whiskers plots. Whiskers represent the maximum and minimum value; the box represents the interquartile range, while the line in the box represents the median. One participant had a very high total and IFN-g CD4 T cell response (4.010% and 3.797%, respectively), and these values are shown individually as dots on the graph. **(B)** Frequencies of distinct subsets of specific CD4 T cells, based on combinations of cytokine expression. After selecting CD4 T cells, boolean gating was utilized to generate 15 distinct cytokine expressing subsets. The Kruskal-Wallis test was used to assess differences between the groups of infants. None of the parameters assessed were different between the groups (all:  $p > 0.05$ ).

#### 4.4.5 Frequency and cytokine profile of BCG-specific CD8 T cells

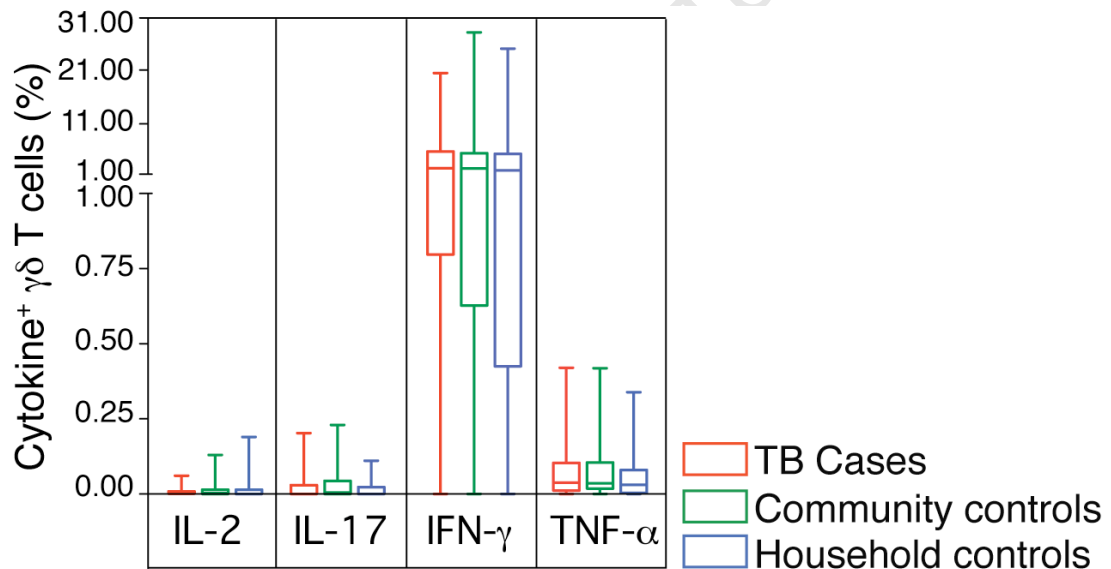
Since CD8 T cells have been shown to be important in protection against TB, we investigated whether frequencies of BCG-specific CD8 T cell responses correlated with risk of developing childhood TB. We found no differences in the frequencies of total cytokine-expressing BCG-specific CD8 T cells within the three groups (**Figure 37A**). The dominant subset comprised total IFN- $\gamma$ -expressing BCG-specific CD8 T cells (**Figure 37A**), however no differences





#### 4.4.6 BCG-induced $\gamma\delta$ T cell response

We assessed whether  $\gamma\delta$  T cell responses may correlate with risk of TB disease following BCG-vaccination of infants at birth. We analysed expression of IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and IL-17 by  $\gamma\delta$  T cells upon BCG stimulation of whole blood from infants in the three groups. In all infants, the  $\gamma\delta$  T cells induced by BCG stimulation almost exclusively produced IFN- $\gamma$ ; as seen for conventional T cell responses, no significant differences were observed within the infant groups (**Figure 38**). A very small  $\gamma\delta$  T cell subset expressed TNF- $\alpha$ , but again, no statistical differences in TNF- $\alpha$  producing  $\gamma\delta$  T cells between the groups were observed. IL-2 and IL-17 expression were negligible in  $\gamma\delta$  T cells (**Figure 38**).

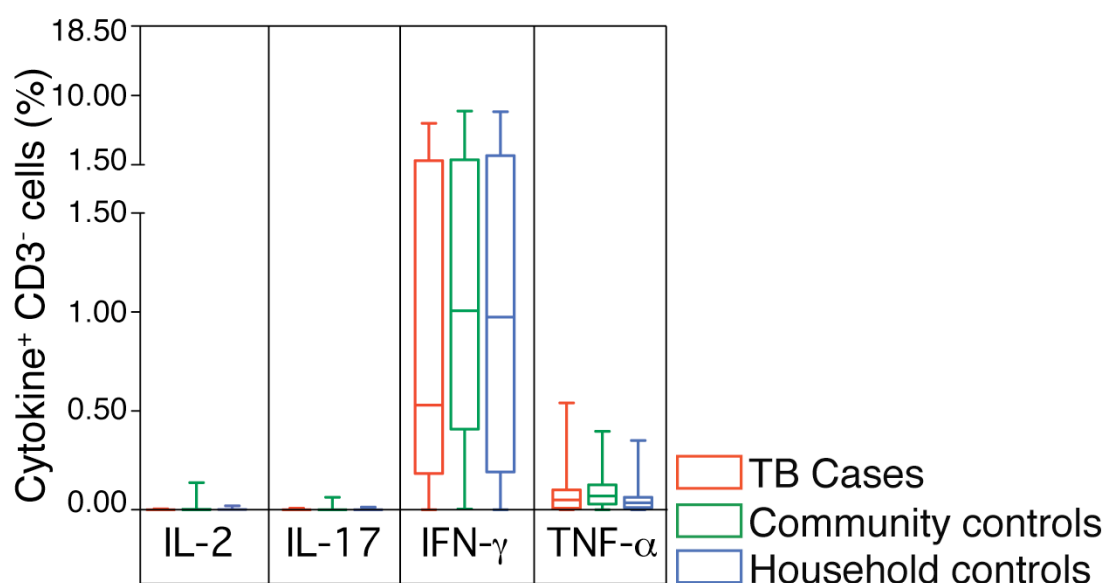


**Figure 38: BCG-induced  $\gamma\delta$  T cell responses.** Frequencies of  $\gamma\delta$  T cells expressing cytokines, following incubation of whole blood with BCG. The Kruskal-Wallis test was used to assess differences between the groups of infants. Results are shown in box and whiskers plots. None of the parameters assessed were different between groups (all:  $p > 0.05$ ).

#### 4.4.7 Comparable BCG-induced CD3<sup>+</sup> responses in the case and control groups

We also observed intracellular IFN- $\gamma$ <sup>+</sup> expression by CD3<sup>+</sup> cells (non-T cell) upon BCG stimulation, and compared the frequencies of these cytokine-expressing cells. The CD3<sup>+</sup> cells are likely to be NK cells (236). We found no

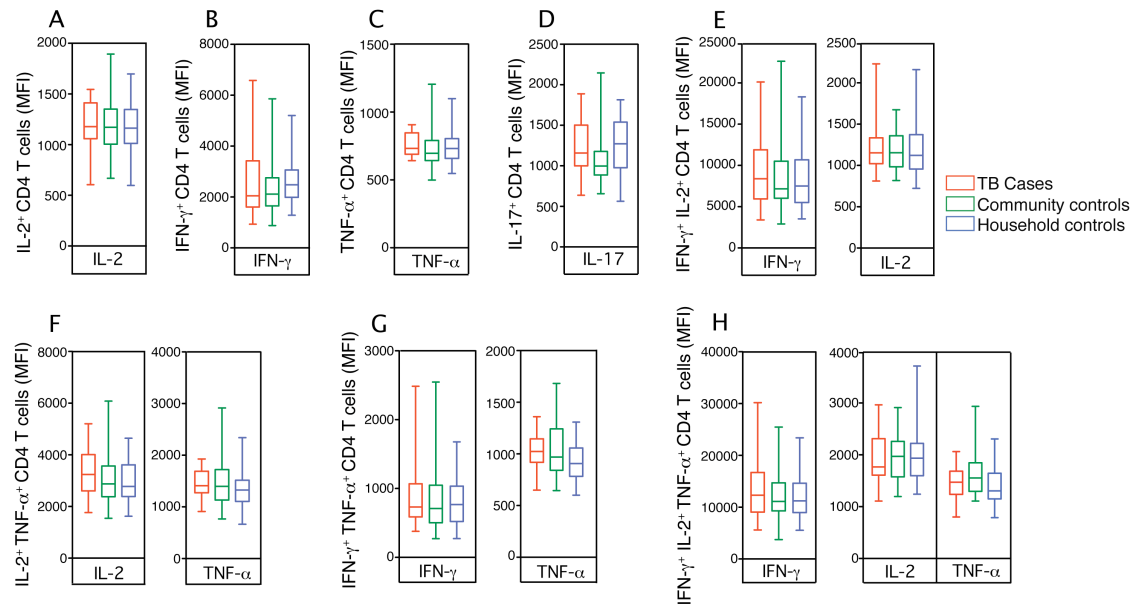
statistical differences in IFN- $\gamma$ -expressing CD3<sup>+</sup> cells between the infant groups (**Figure 39**).



**Figure 39: BCG-induced CD3<sup>+</sup> cell responses in infants from TB cases, community and household controls, following BCG vaccination at birth.** Frequencies of total BCG-induced CD3<sup>+</sup> cell responses detected by a short-term whole blood intracellular cytokine assay are shown. The horizontal lines represent the medians, the boxes represent the inter-quartile ranges, and the whiskers indicate the minimum and maximum values. Results are shown in box and whiskers plots. The Kruskal-Wallis test was used to test for the statistical differences in the frequencies of BCG-specific cells for different subsets.

#### 4.4.8 Median fluorescent intensity of cytokine expression in BCG-specific T cells

Median fluorescence intensity of cytokine expression has been suggested as a useful readout of the “quality” of a T cell response, because the intensity of cytokine expression appears to be highest in polyfunctional T cells (237). We therefore compared the MFI of cytokine expression of BCG-induced CD4, CD8 and  $\gamma\delta$  T cells between the 3 groups. Participants were excluded from analysis if the above-mentioned inclusion criteria were not fulfilled for the flow cytometric data (**Tables 14B-I**). We found no differences in any of the MFI values evaluated between the TB cases and the control groups (CD4 T cell MFI is shown (**Figure 40**)).



**Figure 40: Median fluorescence intensity of BCG-specific CD4 T cells**

CD4 T cells expressing cytokines were selected by boolean gating, after detection with an intracellular cytokine assay, following incubation of whole blood with BCG. The median fluorescence intensities (MFI) of individual cytokines were analyzed, in cells that produced: **(A)** IL-2 only, **(B)** IFN- $\gamma$  only, **(C)** TNF- $\alpha$  only, **(D)** IL-17 only, **(E)** IFN- $\gamma$  and IL-2 together, **(F)** IL-2 and TNF- $\alpha$  together, **(G)** IFN- $\gamma$  and TNF- $\alpha$  together, **(H)** IFN- $\gamma$ , IL-2 and TNF- $\alpha$  together. The Kruskal-Wallis test was used to assess differences between the groups of infants. Results are shown in box and whiskers plots. None of the parameters assessed were different between the groups (all:  $p > 0.05$ ).

## 4.5 Discussion

We report that, following newborn BCG vaccination, the magnitude and profile of cytokine expression of BCG-specific CD4 and CD8 T cells did not show association with risk of childhood TB disease development. Importantly, there were no differences in polyfunctional BCG-specific CD4 T cells, which co-express IFN- $\gamma$ , TNF- $\alpha$  and IL-2. We also confirm production of IFN- $\gamma$  by  $\gamma\delta$  T cells and by CD3<sup>-</sup> cells when whole blood is stimulated with BCG; however, expression of this cytokine by these subsets was not associated with reduced risk of childhood TB.

Multiple experimental studies have shown that the Th1 cytokines, IFN- $\gamma$  and TNF- $\alpha$ , are required for immunity against *M.tb* infection and disease (210, 226, 238). This is supported by findings from experimental TB vaccine

studies that evaluated biomarkers of protection (239-241). For example, in a heterologous prime-boost strategy of BCG followed by adenovirus expressing Ad85A, Forbes *et al.* reported a correlation between magnitude of polyfunctional Ad85A-specific Th1 responses in the lungs after *M.tb* challenge and protection against disease (234). Transfer of ESAT-6-specific Th1 memory cells to recipient mice before *M.tb* challenge enhanced protection, suggesting that the quantity of antigen-specific T cells at the disease site is important (242). In contrast, multiple other experimental studies have shown that IFN- $\gamma$  production at the disease site does not correlate with protection against TB; rather, expression of the cytokine may be a marker of the magnitude of the inflammatory response (243, 244). For example, Mittrucker *et al.* reported no correlation of BCG-induced T cell responses and protection in a mouse TB challenge model (243). Furthermore, in a clinical study, Sutherland *et al.* reported that TB diseased patients had a higher polyfunctional CD4<sup>+</sup> T cell response after overnight stimulation of whole blood with ESAT-6 and PPD compared with healthy individuals with a positive tuberculin skin test (245).

In our study, we did not measure IFN- $\gamma$  at a disease site – this is not possible in healthy 10-week old infants – but in peripheral blood. We found no association between the frequency of BCG-specific Th1 cells and risk of childhood TB disease. We could not evaluate the correlates of protection because all infants in our study had received BCG vaccine. In a setting where TB is endemic, it would not be ethical to have an unvaccinated cohort. We propose that our results are important because this peripheral blood outcome is used to assess vaccine take in most clinical trials of new TB vaccines. The latter studies often focus on the quality of the CD4 T cell response, with the hypothesis that polyfunctionality, i.e., combined expression of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 by individual cells, is a marker of protective immunity. We showed no correlation between polyfunctional BCG-specific CD4 T cell responses in peripheral blood and protection against TB.

In an experimental mouse model with *Leishmania major* infection, Darrah *et al.* reported that MFI of cytokine expression could be used as an additional measure of quality of the T cell response, as polyfunctional cells had the highest MFI of cytokine expression (237). In this study, we assessed the MFI of BCG-specific T cell cytokine expression and showed no association with risk of TB disease.

We evaluated IL-17 expression in CD4 T cells based on evidence that this cytokine plays a protective role against TB (214). This is the first study to demonstrate induction of BCG-specific IL-17 cells in infants, following BCG vaccination at birth; however, frequencies of BCG-specific Th17 cells did not correlate with risk of childhood TB.

We investigated CD8 T cell responses as possible correlates of risk based on the important role of this subset suggested by recent experimental and clinical studies (228, 229). For example, Chen *et al.* reported that depletion of CD8 T cells in BCG-vaccinated rhesus macaques led to a decrease in induced immunity upon subsequent challenge of the animals with *M.tb* (228). Furthermore, Bruns *et al.*, showed that patients undergoing anti-TNF therapy had decreased antimicrobial activity against *M.tb* due to diminished numbers of antigen-specific effector memory CD8 T cells, with an associated increased incidence of TB disease (229). We observed no differences when comparing specific CD8 T cell responses between cases and control infants.

We assessed  $\gamma\delta$  T cell responses based on a report that only  $\gamma\delta$  T cells expanded from PBMC of PPD positive donors incubated with BCG, and not  $\gamma\delta$  T cells expanded by phosphoantigen, were able to inhibit growth of *M.tb* in autologous macrophages (246). However, we found no association between the frequency of BCG-induced  $\gamma\delta$  T cells and reduced risk of childhood TB in our study.

Our results strongly suggest that aspects of antigen-specific CD4 and CD8 T cell immunity, or  $\gamma\delta$  T cell immunity, measured at 10 weeks of age, using

BCG as antigen in this whole blood assay, may not correlate with risk of childhood TB. It is likely that this type of immunity may not correlate with protection against TB either. For future similar studies, it would be ideal to include multiple time points for immunogenicity analysis. We propose time points 6, 10 and 14 weeks, 6, 9 and 12 months are rational to include. From unpublished data by Soares *et al.* in our laboratory, BCG-induced immune response is shown to peak at 6 weeks of age in infants. Time points 6, 10 and 14 weeks of age would allow the investigator to evaluate effector responses. Time points 6, 9 and 12 months would allow the investigator to assess established memory T responses. Therefore, we cannot exclude that these outcomes, measured at multiple time points after newborn BCG vaccination, or using different antigens or assay systems, might correlate with risk of childhood TB.

An infant biomarker study of this size ( $n=5,662$ ) has not been reported, to date; the magnitude of the project required that we limit our blood collection to one practical time point, prior to *M.tb* infection, and at an age before significant exposure to environmental mycobacteria. This was also the reason for selecting a single viable bacterial antigen that can be processed for recognition by a wide range of lymphocytes.

The results generated from using BCG as an antigen are likely to be specific. In chapter five, we showed that responses using BCG in a whole blood assay are detectable at 10 weeks of age only in infants who have been vaccinated at birth, and not in unvaccinated infants (247). Regardless, individual mycobacterial antigens might yield different results.

The T cell response to mycobacteria is complex (248), and involves cytotoxic activity (249, 250), for example, in addition to cytokine production. These additional aspects of T cell immunity might correlate with outcome, while routine vaccine take measurements focus on cytokine production, using an assay like ours. We propose additional approaches are useful. For example, in addition to assessing the frequencies of BCG-specific T cells, evaluation of both the specific and non-specific absolute lymphocytes cell counts would be

useful. This approach may help to control or explain for the different number of total CD4<sup>+</sup> and CD8<sup>+</sup> T cells that are inherently present in a population, which is not possible to account for when the data is analysed as frequencies. Similarly, innate host responses may also be important. We propose that biomarkers of protection against TB may only be unraveled when multiple host factors are examined together in a system biology approach. Ongoing, complementary studies will address whether biomarkers of risk of childhood TB may be identified through other approaches. These include measuring soluble levels of cytokines, chemokines, and growth factors after 7 hours' incubation of whole blood with BCG; the cytotoxic, proliferative, and cytokine expressing capacity of T cells following incubation of PBMC with BCG for 3-6 days; and gene expression profiles in PBMC incubated with BCG for 12 hours.

One big caveat to assays that are hypothesis driven is a selection bias; we select immune parameters that we think are important. In the absence of in depth knowledge on immune parameters mediating protection against TB, such approaches have obvious limitations. Therefore, it is rational to prioritize unbiased approaches that generate high throughput data such as global transcriptome analysis.

We observed potent BCG-specific IFN- $\gamma$  production by CD3<sup>+</sup> cells in our study groups. IFN- $\gamma$  production is a critical event in the immune response to *M.tb*. We speculate that the CD3<sup>+</sup> cells are NK cells (236), which have been shown to mediate killing of *M.tb* in human monocytes (251). In *M.tb* infection, NK cells have been shown to be the early producers of IFN- $\gamma$  that may facilitate a cross talk between the innate and adaptive immune responses (251). Our results showed that the magnitude of IFN- $\gamma$  production by CD3<sup>+</sup> cells was similar in all the three groups of infants and could not be associated with reduced risk of childhood TB.

Overall, our results strongly suggest caution when interpreting T cell immune markers commonly evaluated in new TB vaccine clinical trials. More



importantly, our results indicate that protective immunity against *M.tb* may be very complex, and suggests a need to look beyond the classical Th1 immunity when assessing the efficacy of novel TB vaccines in clinical trials.

#### **4.6 Contributions**

B.M.N. Kagina designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under the supervision of Prof. W.A. Hanekom, Dr. B. Abel, and other members of SATVI laboratory. Study design and sample collection was done by Prof. W.A Hanekom and other members of the SATVI laboratory.

University of Cape Town

## **Chapter 5: Delaying BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T cell response**

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### **5.1 Abstract**

In most tuberculosis (TB) endemic countries, BCG is usually given around birth to prevent severe TB in infants. The neonatal immune system is immature. Our hypothesis was that delaying BCG vaccination from birth to 10 weeks of age would enhance the vaccine-induced immune response.

In a randomized clinical trial, BCG was administered intradermally either at birth (n=25) or at 10 weeks of age (n=21). Ten weeks after vaccination, and at 1 year of age, vaccine-specific CD4 and CD8 T cell responses were measured with a whole blood intracellular cytokine assay.

Infants who received delayed BCG vaccination demonstrated higher frequencies of BCG-specific CD4 T cells, particularly polyfunctional T cells co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, and most strikingly at 1 year of age.

Delaying BCG vaccination from birth to 10 weeks of age enhances the quantitative and qualitative BCG-specific T cell response, when measured at one year of age.

## 5.2 Introduction

Worldwide, more than 100 million children receive BCG each year (221). Studies have shown that neonatal BCG vaccination confers protection against severe forms of childhood TB, i.e. TB meningitis and miliary TB (221, 222). The protection afforded by BCG vaccination against pulmonary TB, the most common form of TB, is variable and mostly poor (50). Several factors have been implicated in variable efficacy of BCG against TB. These factors include the BCG strain, BCG dose, prior exposure to environmental NTM, host genetic variations, *M.tb* strain, vaccination route and schedule (50, 252-254).

Our laboratory has previously studied the effect of BCG strain, vaccination route and host genetics on BCG-induced immunity. For example, a study by Hawkridge *et al.* reported that two different BCG strains and vaccination routes might not influence the immunogenicity and the efficacy levels in infants (101). In adults with history of BCG vaccination, a study by Shey *et al.* showed that SNP on TLR 6 could influence mycobacteria-specific responses after stimulation of whole blood with specific antigens for 20hrs (255). The South African BCG vaccination policy advocates for at birth or soon after birth vaccination schedules. The NTM exposure is unlikely to occur at birth or soon after birth; hence NTM may not influence BCG immunogenicity in South Africa. In this chapter, we chose to assess the effect of vaccination timing on the vaccine take immune responses.

It has been shown that T cell mediated immunity is important against TB. The CD4 T cells that produce the Th1 cytokines: IFN- $\gamma$ , TNF- $\alpha$  and IL-2 are thought to be critical for protection against TB (256, 257). This kind of Th1 response is characteristic following BCG vaccination (130, 258). In areas where TB is highly prevalent, BCG vaccination is widely administered at or soon after birth because of the risk of early exposure to *M.tb* (124). Evidence exists that the Th1 response at birth is “immature”, for example, progressively increasing specific Th1 cell-mediated immunity has been shown when measles vaccine was given at 6, 9 and 12 months of age,

respectively (259). We proposed that allowing maturation of the neonatal immune system to 10 weeks of age prior to BCG vaccination would enhance vaccination-induced T cell immunity.

Administration of BCG vaccine soon after birth improves on the BCG vaccination coverage. Consequently, BCG vaccination coverage in South Africa is very high. For example, in 2005, BCG coverage in the Western Cape of South Africa was 99% (260). One of the caveats to the 10-week delay in BCG vaccination would be an increased chance of infants missing to get the vaccine altogether in their lifetime, which may reduce BCG vaccination coverage. This may result in increased incidence of disseminated TB (261). However, the 10-week delay falls within the schedule when the infants return to health facilities for other EPI vaccines (262). This could reduce the chance of infants missing to get the vaccine by this age. The risk of *M.tb* infection by 10 weeks of age is low as well as the risk of exposure to NTM, which are present in water, soil, and biofilms (263). BCG vaccination in HIV infected infants may cause BCGiosis (156). Neonatal HIV testing at birth is insensitive due to the persistence of passively transferred maternal anti-HIV antibodies. Therefore, the 10-week delay in BCG vaccination may benefit newborns born to HIV infected mothers as their HIV infection could be accurately determined by this age, allowing for an informed decision on whether to administer the vaccine. The effects of optimizing vaccination timing could have important implications for ultimately improving protection against TB disease through BCG vaccination.

Marchant *et al.* reported that varying the age of BCG vaccination did not affect BCG immunogenicity when assessing interferon gamma (IFN- $\gamma$ ) production by PBMC stimulated with mycobacterial antigens (258). Hussey *et al.* also reported that a delay in BCG vaccination from birth to 10 weeks of age did not influence IFN- $\gamma$  secretion, proliferative responses, or cytotoxic potential of BCG-specific T cells, following incubation of PBMC with mycobacterial antigens (136). Since these results have emerged, we have shown that measurement of IFN- $\gamma$  alone underestimates the complexity of

the BCG-induced Th1 response. BCG vaccination in infants induces multiple Th1 subsets, defined by expression of different combinations of IFN- $\gamma$ , TNF- $\alpha$  and IL-2 by specific cells (130). We wished to comprehensively address the effect of age on the magnitude and quality of Th1 immunity induced by BCG in infants. The quality (cells expressing multiple cytokines) of vaccine-specific immune responses is a key outcome in assessing immunogenicity. Evaluation of vaccine-specific cells expressing multiple cytokines has been shown to correlate with protection in different vaccination preventable disease models (264-266).

### **5.3 Materials and methods**

#### **5.3.1 Participant enrolment and follow-up**

Participants were enrolled between April 2006 and March 2008 in Khayelitsha, a suburb of Cape Town with an extraordinarily high TB incidence, reported to be 1,614 per 100,000 in the first quarter of 2008 (Case notification rate, City of Cape Town).

This formed part of a larger study investigating clinical and immunological characteristics of HIV-exposed and unexposed infants. In brief, pregnant women were approached at a public antenatal clinic for enrolment of their infants. For the study reported here, infants who were born to HIV-infected women or women with unknown HIV status, exposed to active TB in the household, born prematurely ( $\leq 36$  weeks gestational age) or had low birth weight ( $\leq 2.5$ kg), or who had significant perinatal complications were excluded. In addition, all infants with a positive IFN- $\gamma$  response to ESAT-6/CFP-10 at 10 weeks of age were excluded (see below for assay details). Infants were randomly assigned during antenatal recruitment to receive BCG (intradermal Danish strain 1331, Statens Serum Institute) on the first day of life ("birth vaccination"), or the identical BCG vaccination at 10 weeks of age ("delayed vaccination"). Infants were followed at 10 weeks, 20 weeks and at 50 weeks of age. A window period of -2 to + 4 weeks around the week 10 visit was allowed (e.g. 8 to 14 weeks). The time period for the 20-week visit ranged from 18 to 28 weeks, and 41 to 54 weeks for the 50-week visit.

Regulatory approval was obtained from the research ethics committees of Stellenbosch University and the University of Cape Town. Written informed consent was obtained from all mothers in their home language. All HIV testing was completed in conjunction with informed consent and pre-and post-test counselling.

### **5.3.2 Blood collection and intracellular cytokine assay**

One mL blood was collected at 10, 20, and 50 weeks of age in heparinized syringes. Whole blood was processed within 2 hours of collection, as previously described (178). Briefly, 250 $\mu$ L whole blood was incubated for 12 hours at 37°C with viable BCG (reconstituted from the vaccine vial, Danish strain, Statens Serum Institute,  $1.2 \times 10^6$  CFU/mL) and the co-stimulatory antibodies anti-CD28 and anti-CD49d (BD Biosciences, 0.5 $\mu$ g/mL each). 250 $\mu$ L blood incubated with SEB (Sigma, 10 $\mu$ g/mL), and 250 $\mu$ L incubated with the co-stimulatory antibodies alone (unstimulated) served as positive and negative controls, respectively. Brefeldin-A (Sigma, 10 $\mu$ g/mL) was added for the last 5 hours of incubation. Following incubation, red blood cells were lysed and white cells fixed with BD FACS Lysing Solution (BD Biosciences), and the cells cryopreserved.

Cryopreserved cells were later thawed and washed in 1% bovine serum albumin (Sigma) in phosphate buffered saline (PBS, BioWhittaker), and permeabilised using Perm/Wash Solution (BD Biosciences). Cells were then stained with the following antibodies: anti-CD3 Pacific Blue (clone UCHT1), anti-CD4 PerCPCy5.5 (SK3), anti-CD45RA PECy7 (L48), anti-IFN- $\gamma$  AlexaFluor 700 (B27), anti-IL-2-FITC (5344.111), anti-TNF- $\alpha$ -PE (MAb11; all from BD Biosciences), anti-CD8 Qdot605 (3B5, Invitrogen) and anti-CCR7 APC (150503, R&D Systems). After washes, cells were acquired on a LSR II flow cytometer (BD Biosciences), for this experiment configured with 3 lasers and 10 detectors, using FACS Diva 6.1 software (the SOP used for this staining is given in details in chapter 2, appendix II). Compensation settings were set using anti-mouse kappa-beads (BD Biosciences), labelled with the respective fluorochrome-conjugated antibodies. Flowjo 8.7.1 (Treestar) was

used to compensate and to analyze the flow cytometric data. Boolean gating was applied to generate combinations of cytokine expressing CD4 and CD8 T cell subsets (**Figure 42B**).

### **5.3.3 Assay of intercurrent *M.tb* infection**

At each time point, whole blood was diluted 1:5 in RPMI-1640 tissue culture medium (Sigma), containing 1% L-glutamine (Sigma), and incubated with ESAT-6/CFP-10 fusion protein (provided by Tom Ottenhoff, Leiden University Medical Centre, 10µg/mL), phytohemagglutinin (PHA, Sigma, 5µg/mL) and SEB (1µg/mL; both positive controls), or no antigen (negative control), in triplicate, in 96-well plates (modified from Black *et al.*) (267). Plates were incubated at 37°C with 5% CO<sub>2</sub> for 7 days, supernatants were harvested, triplicate wells pooled and stored at -80°C. Later, supernatants were thawed, and an IFN-γ sandwich ELISA was used to quantify IFN-γ in the supernatants derived from the 7-day whole blood assay, (BD Pharmingen). A positive IFN-γ response was defined as 62pg/mL, after background subtraction (twice the assay detection limit of 31pg/mL). Infants with evidence of intercurrent *M.tb* infection/ exposure at 10, 20 or 50 weeks of age, were excluded from analysis.

### **5.3.4 Data analysis**

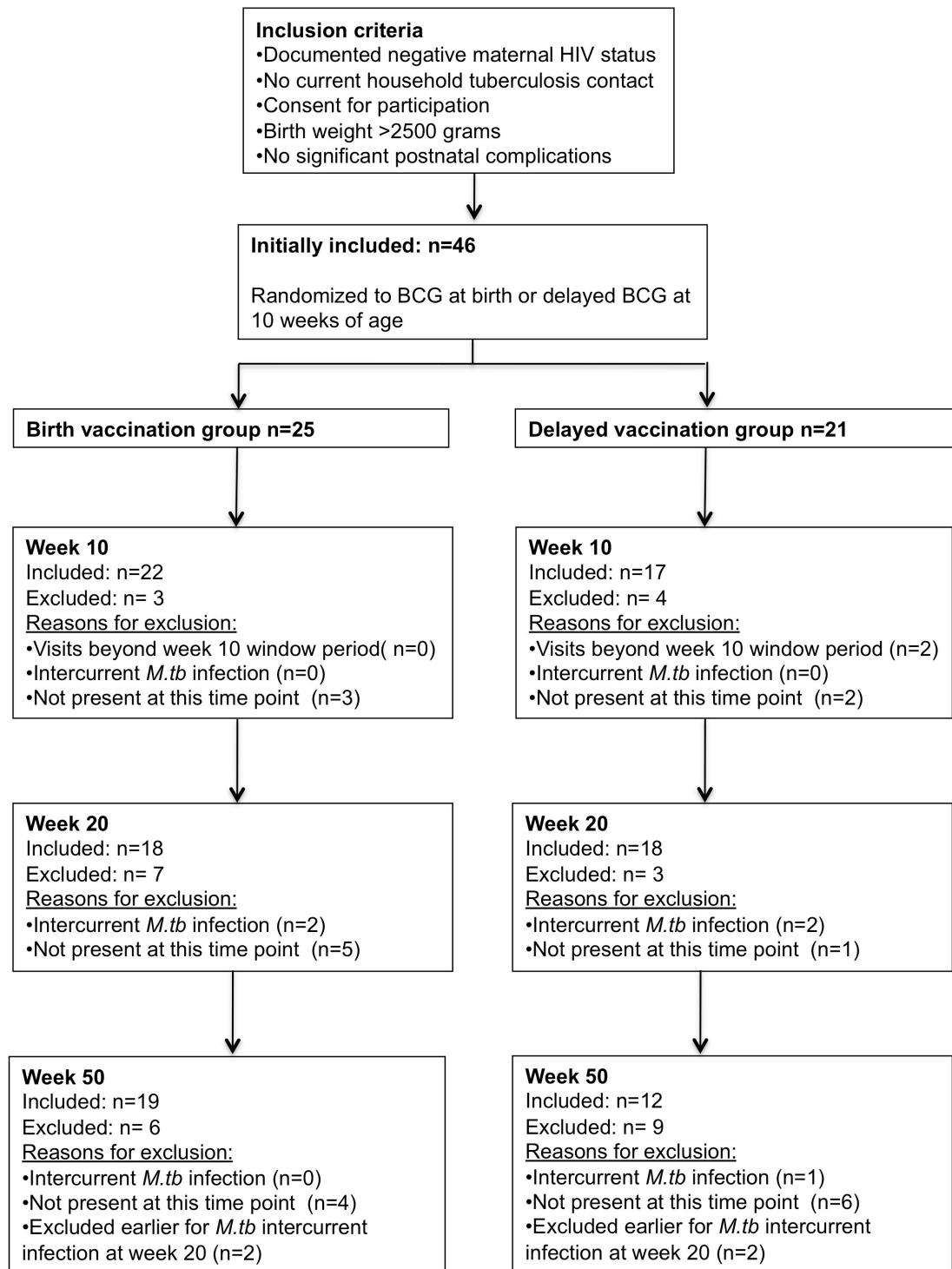
For the intracellular cytokine assay, cytokine expression levels from unstimulated blood were subtracted from levels obtained after BCG or SEB stimulation. (The median level of expression of IFN-γ, IL-2 and TNF among CD4 T cells in unstimulated blood was 0.002%, 0.03% and 0.06%, respectively.) Differences in participant birth weights and gender were assessed using parametric tests. The Mann-Whitney U test was used to assess differences in frequencies of cytokine expressing CD4 and CD8 T cells between the 2 groups. P-values lower than 0.05 were considered to be significant.

## 5.4 Results

### 5.4.1 Participant characteristics

A total of 46 infants were enrolled into this study: 25 in the birth group and 21 in the delayed vaccination group. Infants not present for phlebotomy at a certain time point, bled beyond the defined window period at week 10 visit, or positive for *M.tb* infection were excluded from analysis (**Figure 41**). Results from infants with *M.tb* infection were excluded from the time point at which this was diagnosed, and from subsequent time points. At baseline, no differences were observed in birth weights or gender between the birth and delayed vaccination groups (mean birth weight, (SD): 3306g (435.6) birth vs. 3200g (371.9) delayed;  $p=0.390$ ). Sixteen (64%) infants in the birth group were male compared with 9 (43%) in the delayed birth group ( $p=0.235$ ).

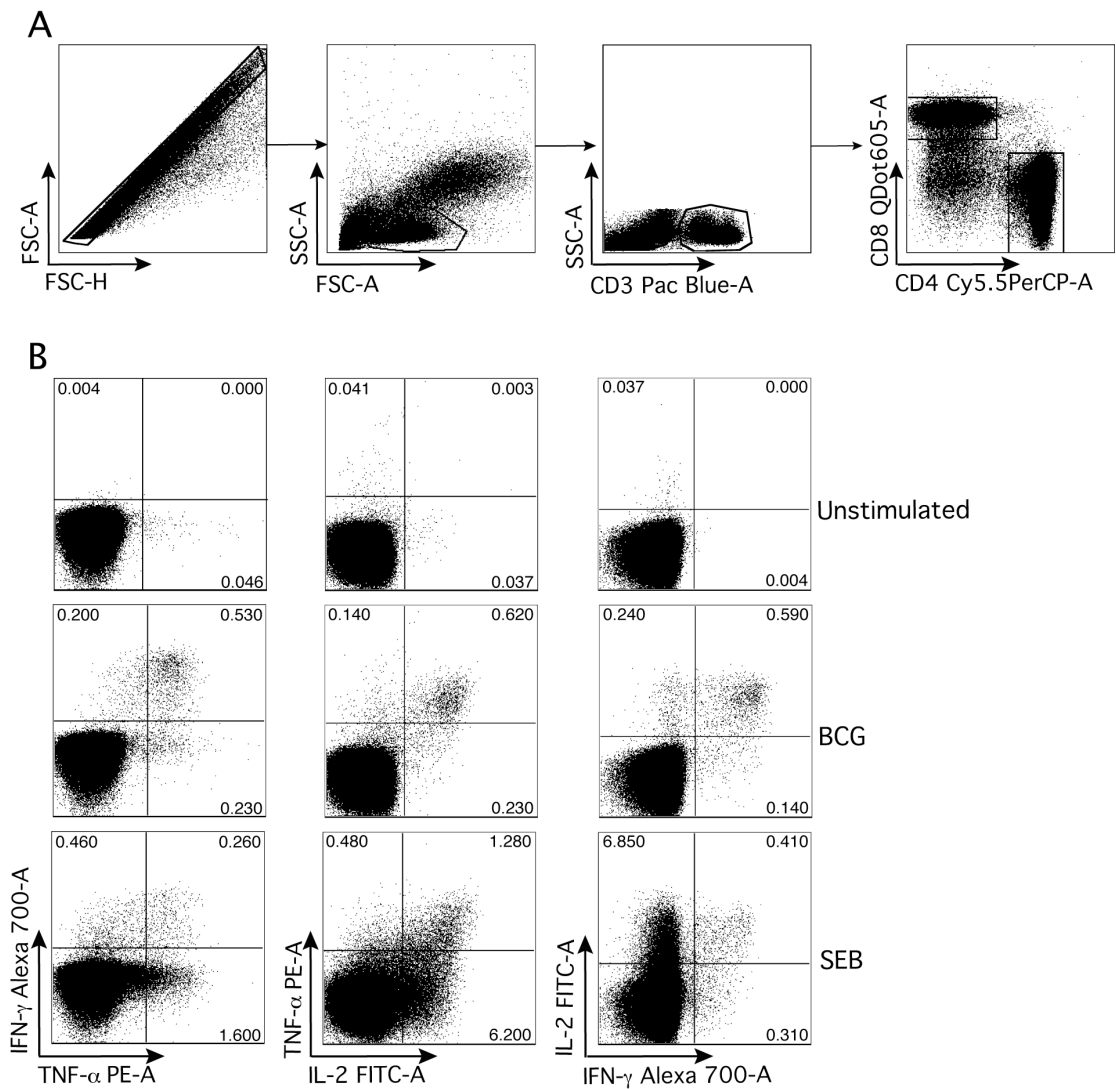




**Figure 41: Overview of eligibility, study flow and participants.** Participants randomized to receive BCG at birth or at 10 weeks were followed up to 1 year of age. Exclusions were made either due to *M.tb* infections and/or absence of the participants at the specified range of time for the blood draw.

#### 5.4.2 Gating strategy for the analysis of BCG-specific T cells

To analyse BCG-specific T cells, we performed ICS as described in the methods section. We first excluded doublets by use of a singlet gate. We then selected for lymphocytes based on the size and granularity characteristics of the cells (SSA vs FSA). We subsequently gated on CD3 expressing cells and further delineated T cells as CD4 or CD8 (**Figure 42A**). Intracellular expression of IL-2, IFN- $\gamma$  and TNF- $\alpha$  were assessed in these T cell subsets. The cytokine expression profiles were assessed in the unstimulated, BCG and SEB conditions, and a representative example is shown (**Figure 42B**). Finally, a boolean gating strategy was applied to assess for the cytokine co-expression patterns.

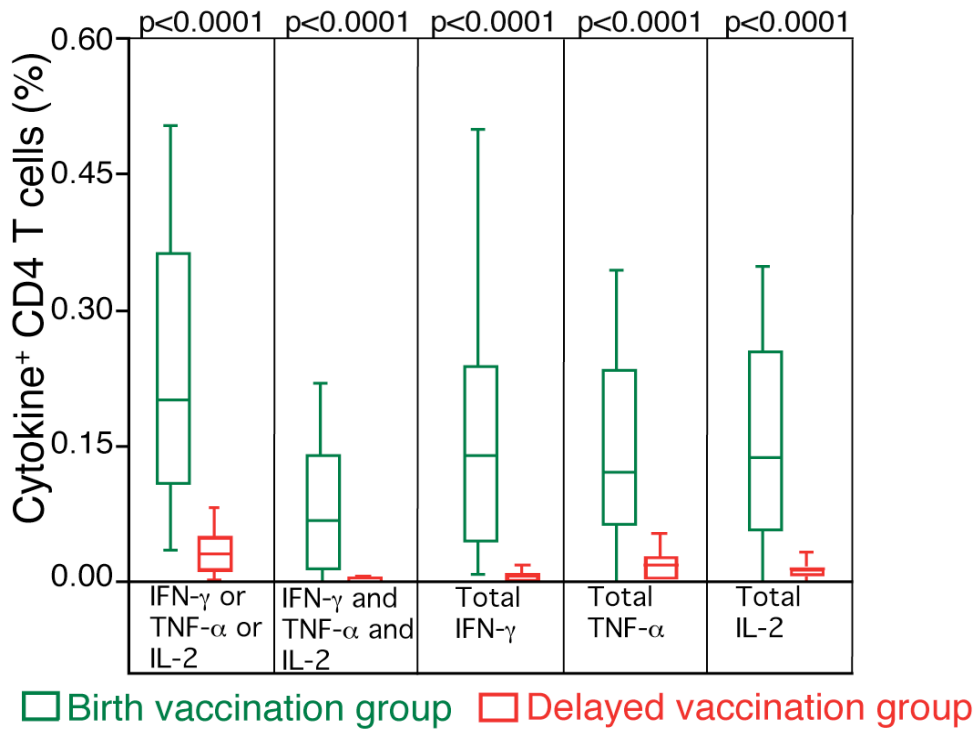


**Figure 42: Gating strategy.** Whole blood was left unstimulated or was incubated with BCG or SEB, and analysed as described in the Methods. **(A)** Doublet cells were excluded by gating on Forward Scatter–Area (FSC-A) against Forward Scatter-Height (FSC-H), followed by lymphocyte selection in a FSC-A against Side Scatter-Area (SSC-A) gate. Subsequently, cell surface antibody markers were used to select for T cells. T cells were selected by gating on CD3<sup>+</sup> events, which were further differentiated into CD4 and CD8 T cells. **(B)** Representative dot plots of cytokine co-expression patterns in CD4 T cells from unstimulated, BCG and SEB-stimulated conditions.

#### 5.4.3 Detection of BCG-specific CD4 T cells using viable BCG as antigen

Before comparing BCG-induced immunity between infants who received birth and delayed vaccination, we evaluated whether incubation of whole blood with viable BCG might result in non-specific T cell activation, or whether a

vaccination-specific response would be detectable. We compared CD4 T cell immunity, measured at 10 weeks of age with an intracellular cytokine detection assay, between participants who had received BCG at birth and those who had not yet received BCG (infants in the delayed group). Cytokine production by CD4 T cells was readily detectable in infants from the birth-vaccinated arm, but was not detectable, or barely detectable in infants who had not received the vaccine at birth (**Figure 43**). The birth-vaccinated infants had high frequencies of BCG-specific polyfunctional CD4 T cells, i.e., CD4 T cells that express IFN- $\gamma$ , TNF- $\alpha$  and IL-2 together, and of CD4 T cells expressing other combinations of Th1 cytokines. Polyfunctional BCG-specific CD4 T cells were absent in infants in the delayed vaccination group. All infants had a positive response to the positive control, SEB. We concluded that T cell responses measured by the short-term whole blood assay system when BCG is used as antigen, are antigen-specific and not due to non-specific T cell activation.

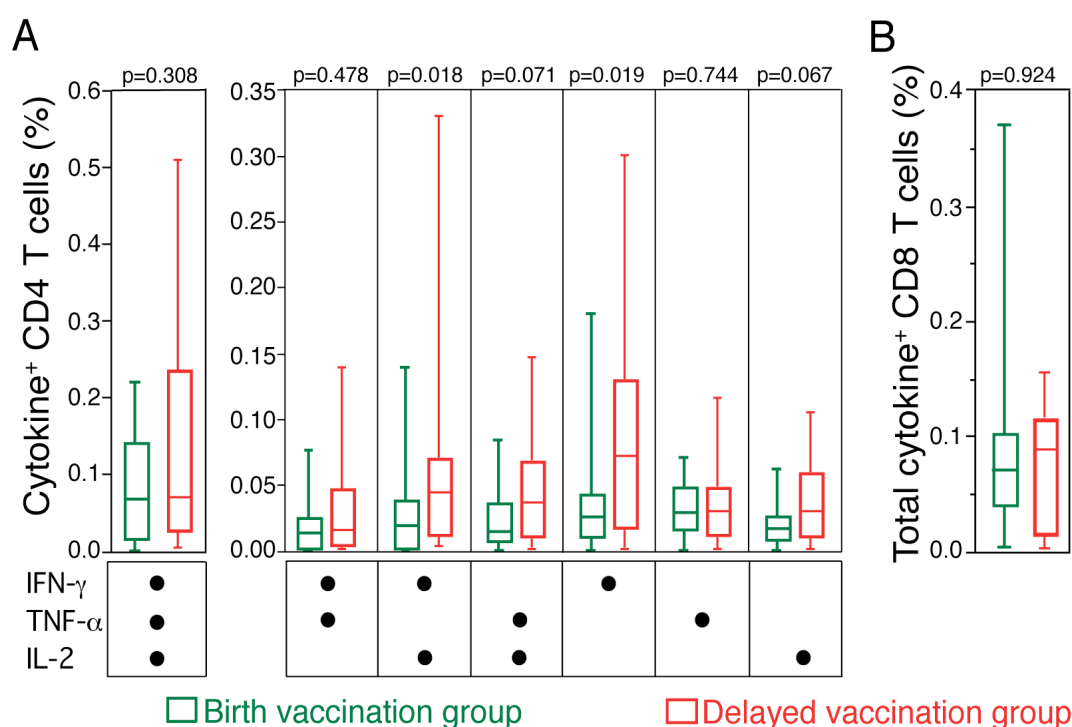


**Figure 43: Comparison of CD4 T cell responses following incubation of whole blood from BCG-vaccinated and unvaccinated infant's blood with BCG, at 10 weeks of age.** When BCG is used as a re-stimulation antigen in the short-term whole blood assay, only CD4 T cells that have previous sensitization to the vaccine respond. The frequencies of cytokine-expressing CD4 T cells detected by a whole blood intracellular cytokine assay (see methods) are shown. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values for differences between the 2 groups.

#### 5.4.4 Delayed vaccination resulted in moderately higher frequencies of BCG-specific CD4 T cells 10 weeks post-vaccination

To assess differences in CD4 and CD8 T cell responses when the vaccine was given at birth or at 10 weeks at age, we measured the Th1 cytokine expression pattern induced by BCG, 10 weeks after vaccination, i.e., at 10 weeks of age in the birth group and at 20 weeks of age in the delayed group. In both groups, high frequencies of polyfunctional CD4 T cells, or cells expressing other combinations of the Th1 cytokines, were observed (**Figure 44A**). The IFN- $\gamma^+$  and IFN- $\gamma^+$ IL-2 $^+$  BCG-specific CD4 T cell subset frequencies were significantly higher in the delayed-vaccination group, compared with the birth-vaccination group (**Figure 44A**). As the frequencies of BCG-specific CD8 T cell subsets were very low, in both vaccination groups, only total

BCG-specific CD8 T cell responses were evaluated: there was no difference between the two groups (**Figure 44 B**). We concluded that delaying BCG vaccination by 10 weeks might result in an increased frequency of BCG-specific CD4 T cells, 10 weeks after vaccination.

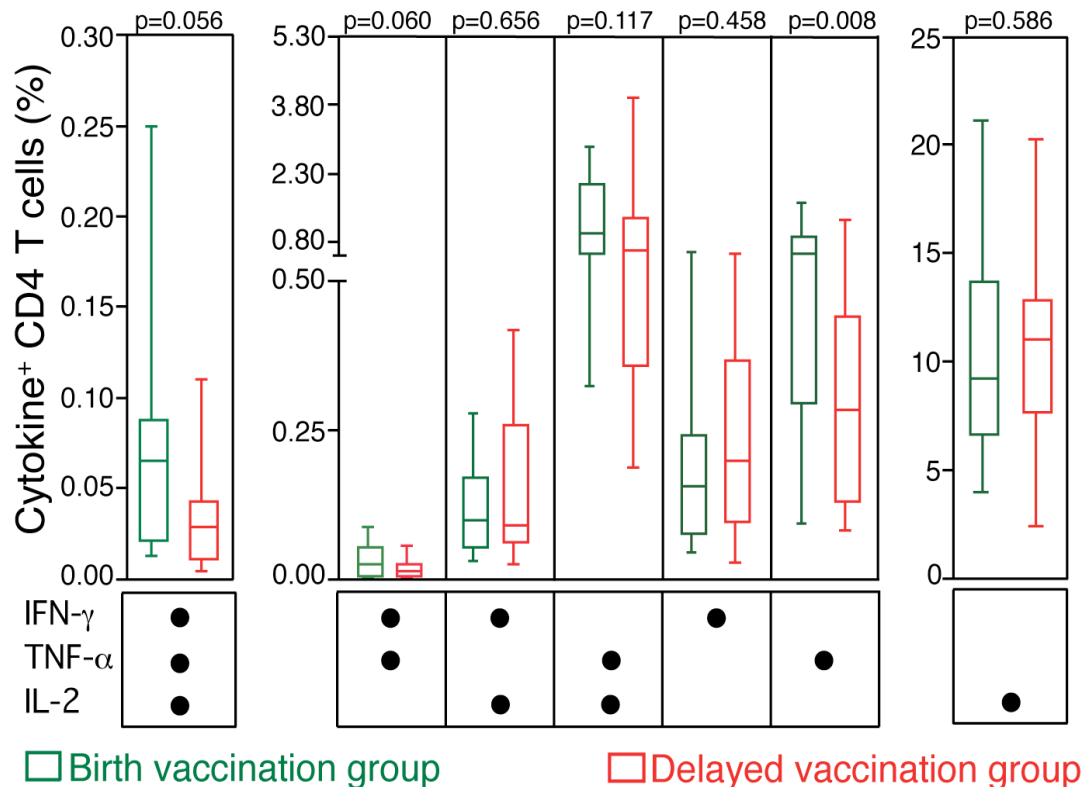


**Figure 44: Comparison of BCG-specific CD4 T cell responses in the 2 groups of infants 10 weeks post-vaccination.** (A) Frequencies of BCG-specific cytokine expressing CD4 T cells are shown, as detected by the whole blood intracellular cytokine assay. (B) Frequencies of all expressing CD8 T cells, evaluated together, after incubation of whole blood with BCG. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values for differences between the 2 groups.

#### 5.4.5 SEB-induced CD4 T cell responses at 10 weeks post-vaccination does not differ with the birth or delayed BCG administration

A positive control in this assay was used to evaluate if the assay was successful. SEB is a superantigen that stimulates T cells via selective crosslinking of the T cell receptor/ CD3 complex with MHC class II molecules irrespective of the cell's previous antigen-specific exposure (268). Thus, the SEB-induced response should be comparable irrespective of the BCG vaccination schedule, in this assay. To confirm this, we therefore evaluated the SEB responses for the two groups at 20 weeks of age. Unlike the BCG-

specific responses that showed moderately higher frequencies in the delayed group at this time point, SEB responses were comparable in the two groups, except the birth-vaccinated group had a higher single TNF- $\alpha$  expressing subset ( $p=0.008$ ) than the delayed group (**Figure 45**). These results further reinforce the validity of the BCG-specific responses, and the differences between the 2 vaccination groups.

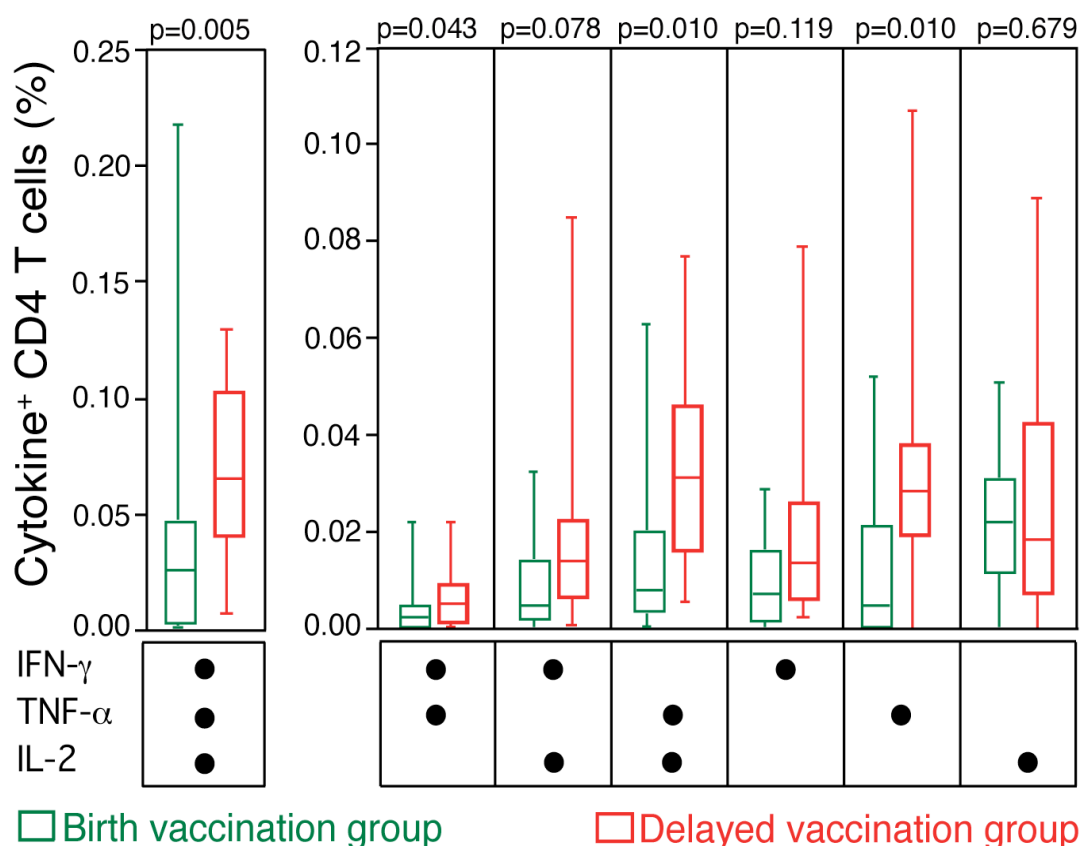


**Figure 45: Comparison of SEB-induced CD4 T cell responses in the 2 groups of infants 10 weeks post-vaccination.** Frequencies of SEB-induced cytokine expressing CD4 T cells are shown, as detected by the whole blood intracellular cytokine assay after incubation of whole blood with SEB. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values of differences between the 2 groups.

#### 5.4.6 Delayed vaccination increased frequencies of polyfunctional BCG-specific CD4 T cells at one year of age

We also compared the BCG-specific memory response at 50 weeks of age. We observed significantly higher frequencies of BCG-specific polyfunctional IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>+</sup> CD4 T cells in the delayed vaccination group, compared

with the birth vaccination group (**Figure 46**). Frequencies of specific T cell subsets co-expressing TNF- $\alpha$  and IL-2, TNF- $\alpha$  and IFN- $\gamma$ , or TNF- $\alpha$  alone, were also higher in the delayed vaccination group. At this age, total BCG-specific CD8 T cell responses were too low for reliable data analysis.

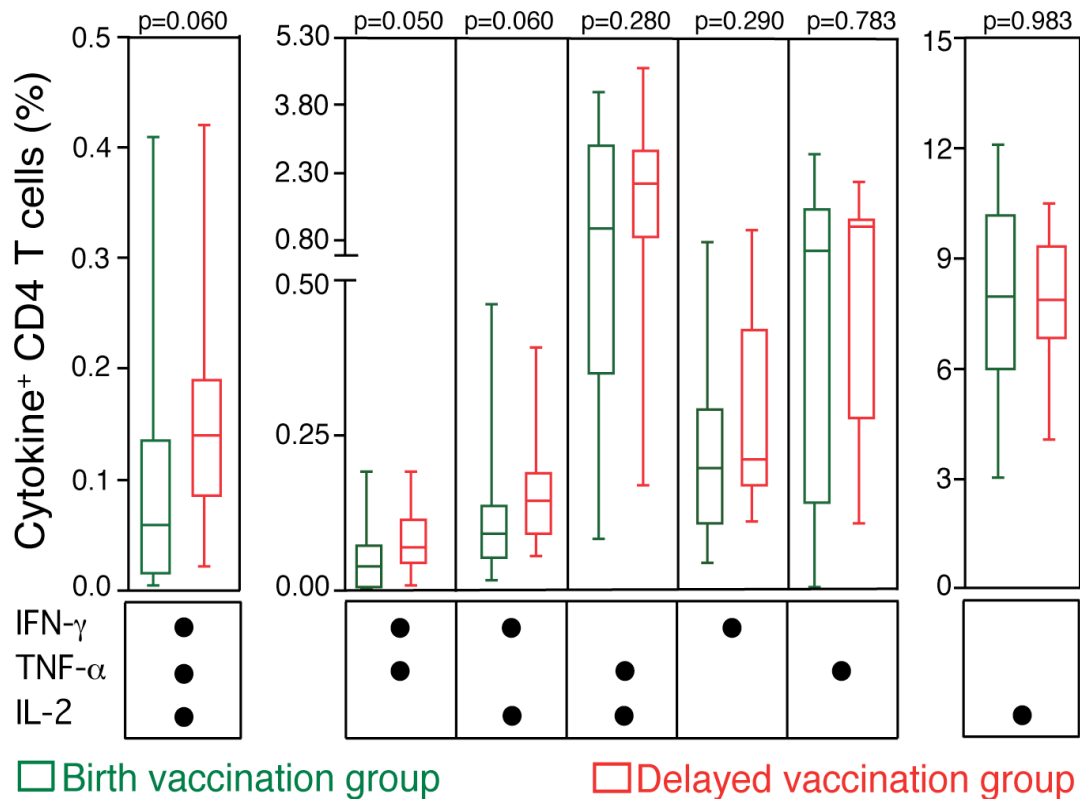


**Figure 46: BCG-specific CD4 T cell responses in the 2 groups of infants at 1 year of age.** Frequencies of cytokine-expressing CD4 T cells detected by a whole blood intracellular cytokine assay are shown. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values of differences between the 2 groups.

#### 5.4.7 SEB-induced CD4 T cell responses at one year of age was comparable in the birth and delayed vaccination groups

When we compared SEB responses between the 2 groups at one year of age, no differences were observed for any of the subsets (**Figure 47**). We concluded that delaying BCG vaccination from birth to 10 weeks of age results in a quantitatively increased and qualitatively more optimal BCG-specific CD4 T cell memory response at one year of age.





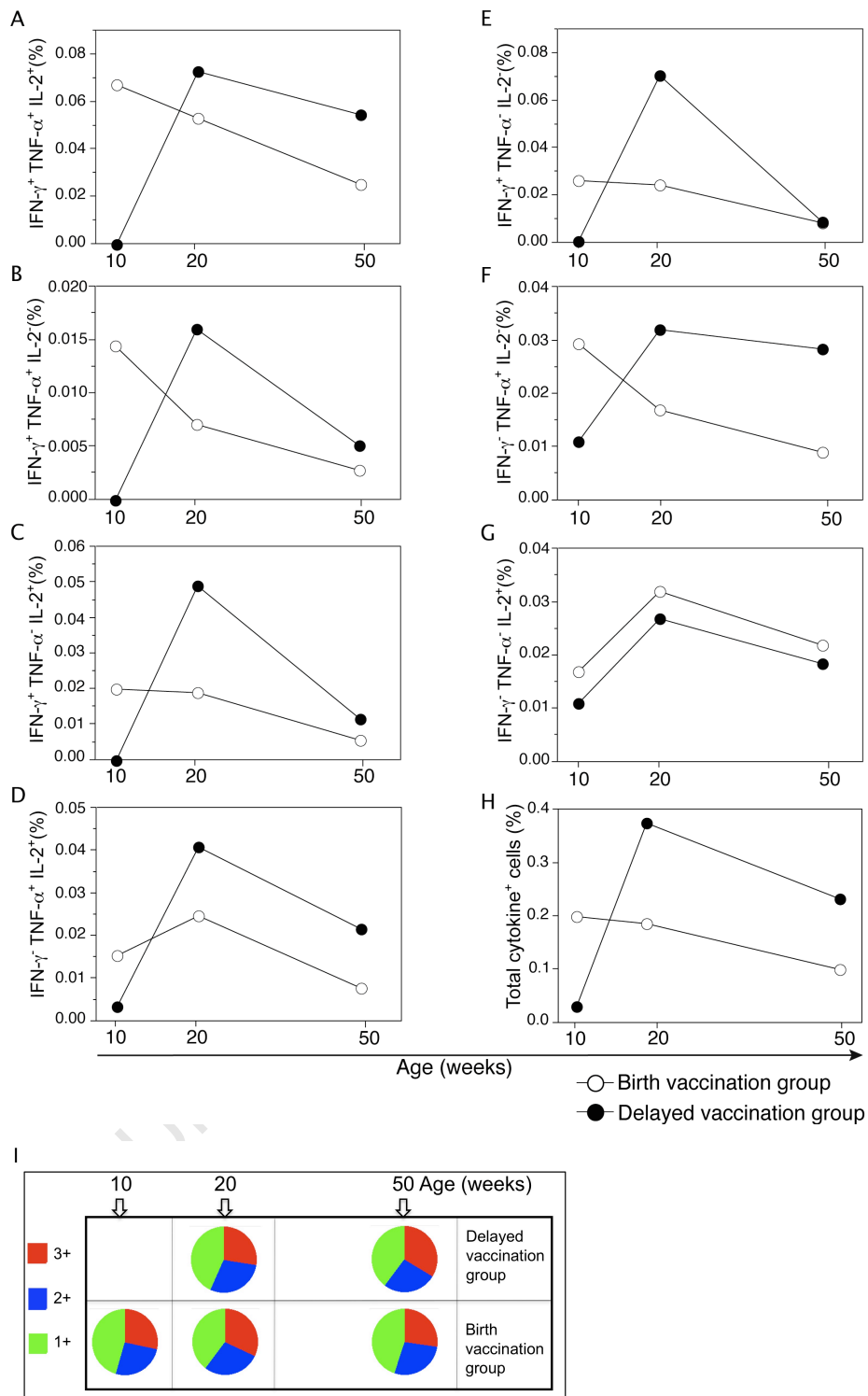
**Figure 47: Comparison of SEB-induced CD4 T cell responses in the 2 groups of infants, at 1 year of age.** Frequencies of cytokine-expressing CD4 T cells detected by a whole blood intracellular cytokine assay are shown for the positive control, SEB, used in this study. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values of differences between the 2 groups.

#### 5.4.8 The kinetics of BCG-specific CD4 T cell response was similar in the birth and delayed vaccination groups

To assess whether the BCG-specific CD4 T cell responses follow a similar kinetic pattern if vaccination is given at birth or at 10 weeks, the total cytokine- and cytokine expression subsets of BCG-specific CD4 T cells were measured longitudinally, at 10, 20 and 50 weeks of age. Total responses are denoted by BCG-specific CD4 T cells that express IFN- $\gamma$ , TNF- $\alpha$ , or IL-2, alone or in combination. Proportions are denoted by the frequencies of polyfunctional (3+ cytokines), bifunctional (2+ cytokines), or monofunctional (1+ cytokines) BCG-specific subsets of all the cytokine expressing cells. In the delayed vaccination group, all of the CD4 subsets peaked 10 weeks post-vaccination, and diminished gradually over the first year of life (**Figure 48A to H**). This pattern was also observed in the birth vaccination group except

for the  $\text{IFN-}\gamma^-\text{TNF-}\alpha^+\text{IL-2}^+$  and  $\text{IFN-}\gamma^-\text{TNF-}\alpha^-\text{IL-2}^+$  BCG-specific CD4 subsets that peaked 20 weeks post-vaccination, and diminished gradually over the first year of life (**Figure 48D and G**). The two subsets that peaked at 20 weeks in the birth group expressed IL-2, a cytokine associated with the maintenance of memory T cells. The proportion of specific polyfunctional T cells in the delayed group increased slightly from 20 to 50 weeks, whilst this proportion decreased marginally in the birth vaccinated group (**Figure 48I, red area of the pie graphs**).

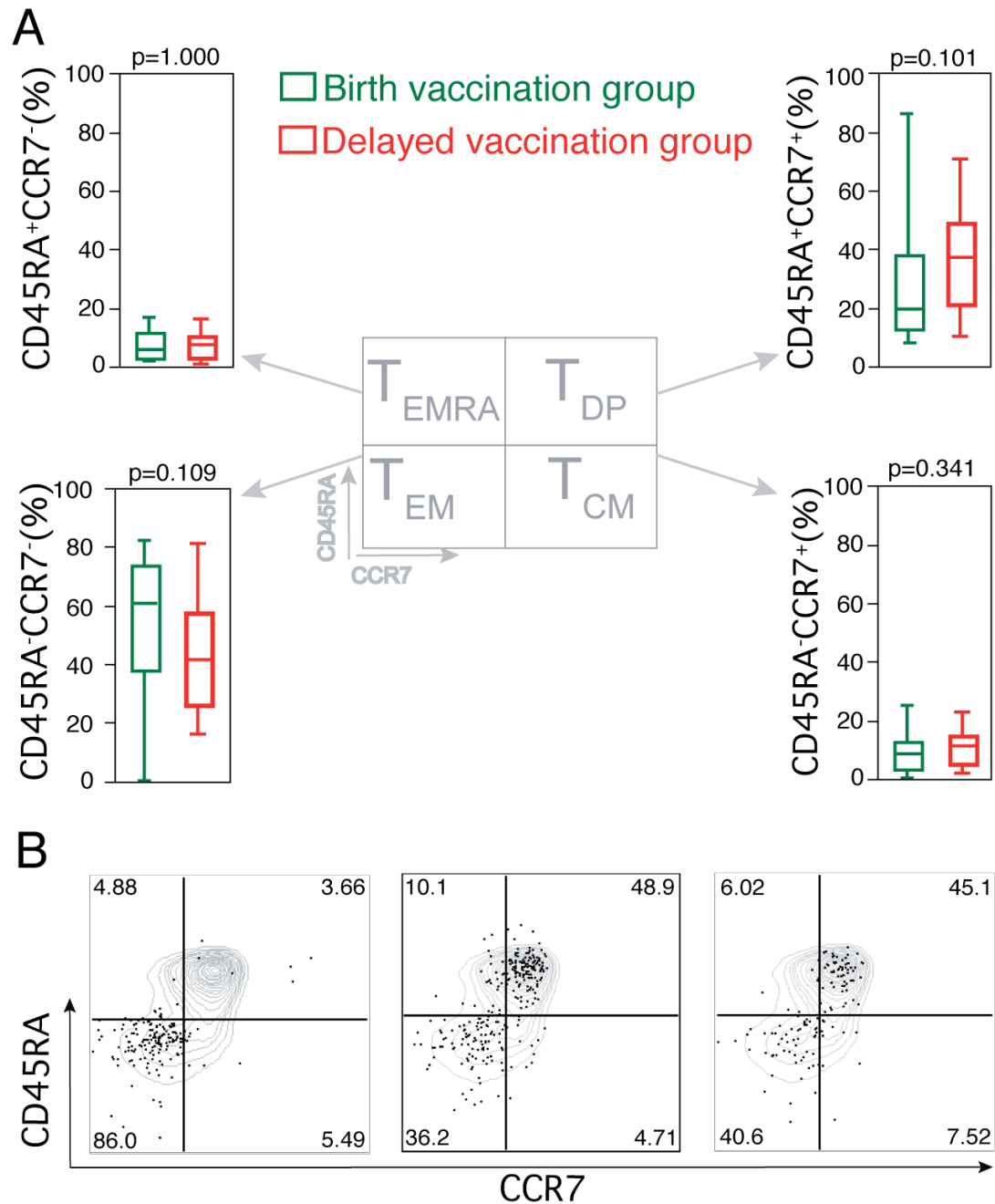
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**Figure 48: Kinetics of BCG-specific CD4 T cells responses in the delayed and birth vaccination groups over one year.** Median frequencies of the profiles of BCG-specific CD4 T cells detected by a short-term whole blood intracellular cytokine assay at 10, 20 and 50 weeks of age. (A) polyfunctional IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>+</sup>, bifunctionals (B) IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>-</sup>, (C) IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>-</sup>IL-2<sup>+</sup>, (D) IFN- $\gamma$ <sup>-</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>+</sup>, monofunctionals (E) IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>-</sup>IL-2<sup>-</sup>, (F) IFN- $\gamma$ <sup>-</sup>TNF- $\alpha$ <sup>+</sup>IL-2<sup>-</sup>, (G) IFN- $\gamma$ <sup>-</sup>TNF- $\alpha$ <sup>-</sup>IL-2<sup>+</sup>, (H) Total cytokines (I) proportions of polyfunctionals (3+), bifunctionals (2+, any two cytokine co-expression) and monofunctionals (1+, any single cytokine expression).

#### 5.4.9 Comparable BCG-specific CD4 T cell memory phenotype between birth and delayed vaccination groups

To assess whether delaying BCG vaccination results in an altered vaccine specific CD4 T cell memory phenotype, we characterized cytokine expressing BCG-specific T cells. The memory phenotype of specific CD4 T cells, as defined by surface expression of CD45RA and CCR7, has in animal models been shown to determine vaccination outcome (269). In both the birth-vaccination and delayed-vaccination groups, effector memory (CD45RA<sup>+</sup>CCR7<sup>-</sup>, T<sub>EM</sub>) T cells predominated at all time points evaluated (**Figure 49** for data at 1 year of age). At one year of age, there was no significant difference for T<sub>EM</sub> or central memory (T<sub>CM</sub>, CD45RA<sup>-</sup>CCR7<sup>+</sup>) in the two infant groups (**Figure 49**).



**Figure 49: Comparison of the memory phenotype of BCG-specific CD4 T cells between the 2 groups of infants, at 1 year of age.** BCG-specific CD4 T cells were identified by cytokine expression using the whole blood intracellular cytokine assay. (A) Box and whisker plots depicting the memory phenotype of all BCG-specific CD4 T cells analysed together. (B) Representative dot plots of the memory phenotype of BCG-specific CD4 T cells (dots), superimposed on the memory phenotype of all CD4 T cells (contour plots). The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. The Mann-Whitney U test was used to calculate p values of differences between the 2 groups.

## 5.5 Discussion

Our study showed that delaying BCG vaccination from birth to 10 weeks of age results in induction of higher frequencies of detectable specific CD4 T cells. In addition, the specific CD4 T cells were more likely to be polyfunctional in the delayed vaccine group, indicating that the “quality” of the BCG-induced response was enhanced.

Our results differ from those reported in two earlier studies, which did not show a difference in T cell immunity when BCG vaccination was delayed (136, 258). Importantly, both those studies used IFN- $\gamma$  measurement alone as outcome. We showed that the most striking differences in induced immunity were at one year of age, highlighting the importance of a longitudinal design of such studies. Data on the kinetics of the BCG-induced immune response in infant cohorts is limited, especially in high TB endemic settings. Our laboratory has ongoing detailed studies aimed to evaluate the kinetics of the BCG-specific T cell response over the first year of life within shorter time intervals.

The immune correlates of vaccination-induced protection against TB are not known. In a 2-year follow up study, we have shown in chapter four that the T cell cytokine expression patterns may not correlate with risk of childhood TB development. However, the frequencies of specific cells, patterns of cytokine production and memory phenotype remain the most widely evaluated outcomes in preclinical and clinical studies assessing immunogenicity of novel TB vaccines. Most vaccinologists would regard a quantitatively greater antigen-specific response as more optimal, following novel TB vaccination in clinical trials. This opinion has been substantiated by multiple animal studies, which have demonstrated that greater frequencies of specific T cells result in improved protection against TB (240-242). In contrast, other studies have shown that the frequency of antigen-specific T cells induced by a TB vaccine may not necessarily correlate with outcome after virulent *M.tb* challenge (243, 270-272). Study design variables, such as the time point evaluated and the compartment analyzed, may be responsible for these discrepant results.

In addition, many of these studies evaluated specific T cells responses by IFN- $\gamma$  production only. Protection against TB without an optimal IFN- $\gamma$  response is not possible. However, it is postulated that measuring other Th1 cytokines in addition to IFN- $\gamma$  would allow a better assessment of the “quality” of the T cell response. For example, presence of polyfunctional T cells, co-expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-2, has emerged as a useful readout of “quality” immune responses (273, 274). In animal models of vaccination against *Leishmania major* (237), and against TB (234), strategies that induce the highest frequency of polyfunctional antigen-specific CD4 T cells are associated with the best protection against subsequent challenge with the pathogen. Further, in HIV-1 infected individuals, slow disease progression is strongly associated with higher frequencies of polyfunctional HIV-specific T cells (275).

Classically, CD45RA and CCR7 have been used to delineate T cells into 4 memory subsets, namely naïve cells (CD45RA<sup>+</sup>CCR7<sup>+</sup>, T<sub>Naïve</sub>), central memory cells (CD45RA<sup>-</sup>CCR7<sup>+</sup>, T<sub>CM</sub>), effector memory cells (CD45RA<sup>+</sup>CCR7<sup>-</sup>, T<sub>EM</sub>), and effector memory cells that have re-expressed CD45RA (CD45RA<sup>+</sup>CCR7<sup>-</sup>, T<sub>EMRA</sub>) (276, 277). Long-lived T<sub>CM</sub> expand in lymph nodes, and differentiate into effector cells (276). In contrast, T<sub>EM</sub> or the more terminally differentiated T<sub>EMRA</sub> populations can immediately home to a disease site for effector functions, but their proliferative capacity and longevity are limited (276). From a hypothetical point of view, induction of longer-lived T<sub>CM</sub> should be a vaccination goal – this view has been substantiated in animal models of macaque infection with simian immunodeficiency virus (SIV) (269). In contrast, induction of T<sub>EM</sub> or T<sub>EMRA</sub> may not be as optimal, as these short-lived populations cannot expand, and are more prone to exhaustion (278). Interestingly, in most studies of human mycobacteria-specific T cells, T<sub>EM</sub> populations predominate and T<sub>CM</sub> are relatively infrequent (130, 212, 230, 279). Our findings corroborate this; however there were no significant differences in these T cell subpopulations upon delaying the BCG vaccination.

Many variables are thought to affect BCG-induced immunity, as described in the introduction. For example, Lalor *et al.* reported that seasonal changes affected the IFN- $\gamma$  response in BCG vaccinated infants, when measured by PPD stimulation of whole blood for 6 days (280). In our study, at 50 weeks of age when the most significant differences in BCG-specific immunity were observed, there was no significant difference in the distribution of birth season between the delayed-and-birth vaccination groups. Further, it is possible that variable exposure to environmental NTM could have confounded our results, although we regard this as unlikely, as all infants were enrolled from the same geographic area. We cannot exclude that other variables could have impacted on the results. At one year, the shorter period post vaccination in the delayed than birth vaccinated groups, may have confounded our results. However, we regarded this time point (one year) as optimal for comparison of established, long-lived (plateau-phase) memory immunity, long after the effector phase.

Vries *et al.* reported that absolute counts of global T and B lymphocytes in healthy infants increased at one and six weeks after birth respectively (220). The same studies reported that over the first year of life, frequencies of memory CD4 and CD8 T cells, defined by the expression of CD45RO remained stable from six weeks of age to one year, but lower than frequencies of the same cell subsets in adults (220). So why did the immune response appear enhanced following delayed-BCG vaccination? The newborn's immune response is often regarded as "immature", when compared with that of adults (135). "Immature" may be a misnomer, as the response may be very appropriate for a baby emerging into a world of continuous antigenic challenge, and where excessive immunity to these stimuli may be detrimental. However, this may result in suboptimal responses to certain vaccines, such as BCG. We propose that the different nature of immunity induced by BCG at 10 weeks of age, compared with birth, could be ascribed to early "maturation" changes in infant immunity. Very limited data on "maturation" of the infant immune response over the first few months of life exists, but factors that may have contributed include inefficient antigen presenting cell function, including limited capacity to produce IL-12 (135), a



critical cytokine for inducing the Th1 responses characteristic of successful mycobacterial immunity. In addition, animal and human evidence suggests that the newborn's immune system is skewed towards a Th2 response (281-283), which may suppress induction of Th1 immunity.

Unlike many infant vaccines that confer increased protection upon boosting, a second BCG vaccination has been shown not to enhance protection (284). New TB vaccination strategies focus on boosting immunity induced by BCG through use of heterologous vaccines. It is therefore critical that we use the prime vaccine most optimally. Our results suggest that the age at which BCG is administered may be a critical variable. The next step would be to confirm these findings in another cohort, prior to larger studies to assess whether delayed vaccination leads to increased protection against TB disease. Makerere University, in collaboration with our laboratory, has enrolled birth- and delayed BCG vaccination cohorts in Uganda, and studies are ongoing to compare the BCG-induced immunity from these cohorts, using more detailed assays. Should this study show similar results of enhanced immunity following delayed BCG vaccination, comprehensive assessment of the implications of delaying BCG vaccination to 8-14 weeks of age, when other childhood vaccines are given routinely, would be warranted.

## **5.6 Contributions**

B.M.N. Kagina designed the experiments, conducted the laboratory and data analysis, and wrote this chapter under supervision of Prof. W.A. Hanekom, Dr. B. Abel, and other members of SATVI laboratory. A.C. Hesselning and other members of Stellenbosch University did study design, sample collection and performed the assay of intercurrent *M.tb* infection.

## **Chapter 6: General Conclusions**

We showed that long-term cryopreservation of fixed white blood cells from whole blood incubated with mycobacterial antigens immediately after collection does not compromise the detection of specific immunity with ICS. We also showed that mycobacteria-specific immunity, measured 10 weeks after newborn vaccination with BCG with this whole blood ICS assay, does not correlate with ultimate risk of developing childhood TB. Finally, we reported that delaying BCG vaccination schedule from birth to 10-weeks of age resulted in enhanced BCG-induced immunity. These results are highly significant to the field of TB vaccinology for several reasons.

First, all our results were generated utilizing a short-term whole blood ICS assay, which is a commonly used method in the measurement of immunogenicity elicited by novel TB vaccines. All our blood samples were stimulated immediately after collection, and then processed followed by cryopreservation. This is similar to what is routinely done in TB vaccine clinical trials. Our results showed that stimulated fixed white blood cells retrieved after long-term cryopreservation are reliable for retrospectively addressing immunological questions. It is possible that novel TB vaccine candidates will demonstrate efficacy prior to identification of immune correlates of protection against TB. In such a scenario, retrospective analysis of samples collected and cryopreserved during the clinical trial period, would be invaluable.

There may also be limitations to the approach of immediate incubation and cryopreservation of fixed cells. This assay system does not allow flexibility in testing new antigens later. Future studies are important to investigate the feasibility of cryopreserving unstimulated whole blood (in addition to the current standard of PBMC cryopreservation), which would allow for the flexibility to introduce re-stimulation antigens post thaw. We propose that cryopreservation is a necessary aspect of vaccine development process that human research ethicists, vaccinologists and immunologists need to maximally utilize when conducting clinical trials.

Secondly, we report that the immunological outcomes routinely evaluated in novel TB vaccine clinical trials, including BCG-specific polyfunctional CD4 T cells, may not correlate with risk of childhood TB development. Additional immunological outcomes should thus be considered for novel vaccine trials. Although specific polyfunctional T cells did not associate with the risk of developing TB in infants, these cells may be an essential component of TB immunity. In a clinical setting, unpublished data from our laboratory show that mycobacteria-specific polyfunctional T cells increase during TB treatment, suggesting emergence during recovery of the host response (Day *et al.*, unpublished data). Conversely, Sutherland *et al.* has reported presence of higher frequency of mycobacteria-specific polyfunctional T cells in patients with TB disease, compared with persons with latent infection (285). Therefore, the association between risk of developing TB disease and presence of mycobacteria-specific polyfunctional T cells remains unclear. We hypothesize that the risk of developing or not developing TB may involve a balance between host inflammation and immune regulation. Decoding the critical components of an optimal balance between these two arms of the immune response within a host may provide elucidate immune correlates of risk of TB disease. Until we have validated correlates of protection against TB disease, our whole blood ICS outcome should best be termed “vaccine take” in these trials. Correlates of protection may be defined only in a randomized controlled trial of an effective vaccine (99). In the interim, studies of correlates of risk of TB disease, such as the study reported here, may require complementary and novel approaches for success. We propose that we should complement hypothesis-driven approaches, such as the current study, which focused on T cell knowledge, with data-driven approaches, which are not restricted by our limited knowledge. Examples of data driven approaches include whole genome expression screens, proteomic analysis and metabolomics (103). The results obtained from these approaches should generate new hypotheses, including novel T cell approaches.

Finally, we report that BCG immunogenicity is enhanced when the vaccine is delayed by 10 weeks after birth. Most novel TB vaccine strategies involve boosting immunity primed by BCG; although alternate priming vaccines are

under development, most are far from clinical application. In the interim, we propose that studies should be conducted to determine the most optimal manner to prime immunity with the current BCG. We are currently testing, in another cohort from Uganda, whether our result of “better” priming when the vaccination is delayed, can be confirmed. A positive finding might have to lead to studies to determine the exact age at which BCG should be given, and these results might have far reaching implications on vaccination policy. Additionally, these findings may be encouraging to the public health sector in developing countries where home births are common, and BCG vaccination at birth is not feasible, for practical and logistical reasons. For example, prior to routine introduction of antiretrovirals in South Africa early after diagnosis of HIV infection in infants, an estimated 110-417 cases of BCGosis were reported per 100,000 BCG vaccinations (156). These findings have resulted in a revised recommendation by the WHO Global Advisory Committee on Vaccine Safety to not routinely vaccinate infants born to HIV-infected mothers at birth (286). This policy is difficult to implement in developing settings; therefore, babies born to HIV-infected mothers will benefit from delayed vaccination – infants who are HIV-infected may then be excluded.

Immunological outcomes reported in this thesis indicate that BCG-induced immune responses are highly variable between individuals. Our group is hypothesizing that this variability may indicate differential host responses to BCG. This means that examining all infants together, without taking into account heterogeneity in responses, may mask correlates of risk that may be present in some groups of infants. Other investigators in our group are currently addressing these hypotheses, primarily through studies of gene expression. Findings from these analyses will contribute significantly to the field of TB vaccinology.

## **6.1 Contributions**

B.M.N. Kagina wrote this chapter under supervision of Prof. W.A. Hanekom and Dr. B. Abel.

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